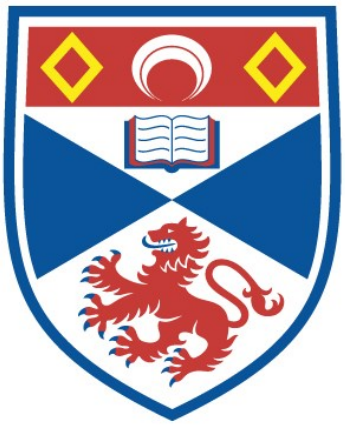
HYPERSENSITIVITY OF ATAXIA TELANGIECTASIA CELLS TO DNA DOUBLE STRAND BREAKS

Nan Liu

A Thesis Submitted for the Degree of PhD at the University of St Andrews



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Thesis submitted for the degree of Doctor of Philosophy to the

University of St. Andrews

by

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ABBREVIATIONS

3AB	3-aminobenzamide
46BR	Fibroblast cell line derived from an patient with immunodeficiency disorder
4-NQO	4-nitroquinoline-1-oxide
AFP	Alphafetolprotein
Ara A	9-β-D-arabinifuranosyladenine
Ara C	9-β-D-arabinofuranosylcytosine
Ara AMP	9-β-D-arabinifuranosyladenine 5′-monophosphate
Ara ATP	9-β-D-arabinifuranosyladenine 5′-triphosphate
AT	Ataxia telangiectasia
AT5BIVA	SV_{40} virus transformed fibroblast cell line derived from an AT homozygous individual
AT-KM	Epstein-Barr virus transformed lymphoblastoid cell line derived from a female AT homozygote
AT-PA	Epstein-Barr virus transformed lymphoblastoid cell line derived from a female AT homozygote
BN	Binucleated (cells)
BrdU	5-bromodeoxyuridine
Bq	Becquerel; unit of radioactivity = 1 disintegration per second
BSA	Bovine serum albumin
CA	Chromosome aberrations
CYT-B	Cytochalasin B
CHO	Chinese hamster ovary cell line
CYT-B	Cytochalasin B
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
dsb	Double strand break in DNA
dpm	Disintegration per minute
E. coli	*Escherichia coli*
EB	Ethidium bromide
EBV	Epstein barr virus
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis (b-aminoethylether) N,N,N′,N′-tetraacetic acid

EM9	EMS and X-ray sensitive mutant of CHO cell line
EMS	Ethyl methane sulphonate
EMU	Ethyl nitrosourea
ESS	Endonuclease sensitive sites
exr$^-$	Excision repair deficient
exr$^+$	Excision repair proficient
F-ara A	9-β-D-arabinifuranosyl-2-fluoroadenine
gpt	A bacterial gene encoding for xanthine guanine phosphoribosyl transferase (XPRTase, EC.2.4.2.22)
GM8505	Fibroblast cell line derived from a Bloom's syndrom patient
Gy	Gray, unit of radiation absorbed dose = 1 joule per Kilogram
^{3}H-TdR	Tritiated thymidine
HBSS	Hank's balanced salt solution
HBSS/BSA	1% (w/v) BSA in HBSS
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HU	Hydroxyurea
irs	γ-ray sensitive mutants of V79 cell line
Ki	inhibition constants
Km	Michaelis constants
Kv	Kilovolts
LET	Linear energy transfer
L5178Y	Mouse lymphoma cell line
μCi	Micro Curie; 1 μCi = 3.7 x 10^4 disintergration per second
min	Minute
MMC	Mitomycin C
MMS	Methylmethane sulphonate
Mn	Micronucleus
MNU	Methyl nitrosourea
MNNG	N-methyl-N'-nitro-N'-nitrosoguanidine
MRC5V1	SV_{40} virus transformed fibroblast cell line derived from a normal human individual
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
N-SW	Epstein-Barr virus transformed lymphoblastoid cell line derived from a normal human female individual
PBS	Phosphate buffered saline

pBR322	Plasmid purified from *E. coli* RR1
PCC	Premature chromosome condensation technique
PCNA	Proliferating cell nuclear antigen
PFGE	Pulsed field gel electrophoresis
PLD	Potentially lethal damage
PMFS	Phenylmethylsulphonyl fluoride
pol	Polymerase
pSV2 *gpt*	A recombinat plasmid containing SV40 sequencies and the bacterial gene *gpt*
RBE	Relative biological effectiveness
RE	Restriction endonuclease
RNase A	Ribonuclease A
RAG-1, RAG-2	Recombination activating gene 1 and 2
rpm	Revolutions per minute
RSS	Recombination signal sequence
SCE	Sister chromatid exchanges
ssb	Single strand breaks in DNA
SLD	Sublethal damage
SV_{40}	Simian virus
TCA	Trichloroacetic acid
Topo	Topoisomerase
UDS	Unschedualed DNA synthesis
UV	Ultraviolet radiation
V79	Chinese hamster lung fibroblast cell line
V(D)J	Variable, diversity, joining recombination
V-C4, V-E5 , V-G8	X-ray sensitive mutants of V79 cell line
XR-1	X-ray sensitive mutant of CHO cell line
XR-V9B	X-ray sensitive mutant of V79 B cell line
xrs	X-ray sensitive mutants of CHO cell line

ABSTRACT

Cells of ataxia telangiectasia (AT) individuals are hypersensitive to a variety of DNA damaging agents such as ionizing radiation and bleomycin, presumed to be due to an intrinsic defect in repair of DNA damage. The nature of the DNA lesion(s) to which AT cells are abnormally sensitive, and the defect in DNA repair are presently unclear. The major part of this project aimed at investigating the sensitivity of AT cells to DNA double-strand breaks (dsb) generated by restriction endonucleases (RE), thereby verifying the hypothesis that AT cells are deficient in the processing of dsb.

AT lymphoblastoid cell lines (AT-PA and AT-KM) used in this study were initially characterized and found to be approximately 3 times more sensitive to ionizing radiation in the induction of micronuclei (Mn) and chromosomal aberrations (CA) compared with a normal lymphoblastoid cell line (N-SW). Other cellular characteristics were observed in AT-PA cells following γ-irradiation such as normal induction and rejoining of dsb and reduced inhibition of DNA synthesis.

By using SLO poration, RE were introduced into the AT and normal cell lines and the yield of CA resulting from RE-induced dsb were subsequently investigated. The frequencies of CA induced by *Pvu* II were 2 - 4 fold higher in AT-PA than in N-SW cells at both 5 h and 24 h sampling times. The enhanced frequency of CA in AT cells treated with *Pvu* II was principally a result of an increase of chromatid aberrations, rather than chromosome aberrations at 24 h. Higher frequencies of chromatid exchanges appeared in AT-PA than in N-SW cells. The results suggest that AT cells are characterized by a defect in dsb processing that converts a higher number of dsb into CA than in the normal cell line.

With respect to the different end-structures of RE-induced dsb, cohesive-ended dsb generated by *Bam*H I and *Pst* I were found to induce lower frequencies of CA than blunt-ended dsb generated by *Pvu* II and *Eco*R

V in both the AT cell lines and the normal cell line. The results support the previous observations that cohesive-ended dsb are less clastogenic than blunt-ended dsb (Bryant 1984). Although inducing lower frequencies of CA than *Pvu* II and *Eco*R V, *Bam*H I and *Pst* I induced higher number of CA in both AT-PA and AT-KM cells when compared with N-SW cells, again indicating a defect in processing cohesive-ended dsb exists in AT cells.

A potent DNA repair inhibitor, Ara A, was found to potentiate the production of CA by RE in AT and normal cells. The enhancement ratios (by ara A) for CA induced by *Pvu* II and *Pst* I were higher in N-SW cells than in AT-PA and AT-KM cells. Ara A appeared to have no effect on the frequencies of CA induced by *Bam*H I in any of the cell lines tested. Based on these findings, a mechanism for the rejoining of RE-induced dsb in which DNA repair synthesis may be involved is proposed, and it is postulated that dsb in AT cells are subjected to greater end degradation.

Inhibition of DNA synthesis was observed in normal cells after treatment with *Pvu* II and *Eco*R V, while *Eco*R I and *Bam*H I had only minor effect. AT-PA cells were found to be resistant to RE-induced inhibition of DNA synthesis, as in the case of ionizing radiation. This result suggests that RE-induced blunt-ended dsb mimic radiation-induced lesions in supressing DNA synthesis in normal cells and that AT cells respond to RE-induced dsb in a similar way to damage induced by ionizing radiation.

Finally, when a nuclear extract from N-SW cells was introduced into *Pvu* II-treated AT-PA cells, it was able to confer a normal frequency of CA. In contrast, neither whole cell nor nuclear extracts from normal cells influenced the production of CA induced by γ-rays. These findings provide evidence for the presence of factor(s) in normal nuclear extract which complements the defect in processing of RE-induced dsb in AT cells.

Chapter I

Introduction

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1. 1. Ataxia telangiectasia

1. 1. 1. Clinical features

Ataxia telangiectasia (AT) is a human autosomal recessive disorder which occurs in early life (Border and Sedgwick 1958, Boder 1985). The incidence of the inherited disease is estimated to be 1/40,000 (Sedgwick and Boder 1972). The clinical features of AT include a progressive cerebellar ataxia which is apparent at an early age, and telangiectasia shown as a dilation of venous capillaries in the conjunctiva of the eyes, in the ears and in the neck. (Boder and Sedgwick 1958). Other clinical manifestations of AT are progressive neuromotor dysfunction and immunological deficiency (Boder and Sedgwick 1958, Boder 1985). Patients with AT frequently have an abnormally small or absent thymus and abnormally low levels of immunoglobulins IgA, IgE and IgG in serum and external secretions, a phenomenon that may result from a defect in the synthesis of these immunoglobins (Waldmann 1982). As a result of deficiencies in immunity, AT patients are subject to recurrent infections, particularly sinopulmonary infections. Epidemiological studies point to a high cancer-proneness of AT; appoximately 10% of AT patients develop a malignancy in childhood (Spector et al 1982, Peterson et al 1992). Radiotherapy of AT cancer patients revealed an unusual and sometimes catastrophic overreaction to a conventional dose of ionizing radiation (Gotoff et al 1967), a feature of hyper-radiosensitivity which has been found to be uniformly present in all AT individuals. Some of the symptoms, e.g., immunoglobulin abnormalities, neuronal degeneration, elevated level of serum alphafetoprotein (AFP) produced in immature liver, and the prematurely aged appearance of AT patients, are suggestive of a fault in tissue differentiation. Nevertheless, the relationships between these clinical

features are not clearly understood. The primary deficiency in the molecular aspects leading to all these pathological changes is of great interest across a wide range of disciplines. A large amount of research has been focused on the multifaceted nature of the response of AT cells to DNA damage, e.g., that induced by ionizing radiation (see review of McKinnon 1987).

1. 1. 2. Cellular sensitivity to ionizing radiation and DNA damaging agents

Sensitivity to radiation

Cultured AT cells from afflicted individuals are extremly sensitive to ionizing radiation. The cell killing effects of irradiation are 2 to 4 times higher in AT cells than in normal cells when examined by their colony-forming ability (Taylor et al 1975, Cox et al 1978, Paterson and Smith 1979). However, AT cells are generally no more sensitive to ultraviolet (UV) irradiation (Paterson and Smith 1979, Scudiero 1980, Arlett et al 1982) than normal cells, although it has been reported that AT cells show abnormally high sensitivity to near-UV at approximately 320 nm (Paterson and Smith 1979).

Enhanced lethality appears in AT cells following irradiation under either aerobic or hypoxic conditions. The oxygen enhancement ratio (OER) is found to be similar for normal and AT cells (Ritter et al 1979, Paterson et al 1979, Paterson and Smith 1979) and this suggests that the nature of damage induced by ionizing radiation may not differ between AT and normal cells. With respect to the response to irradiation at low dose-rate, the clonogenic capability of normal human cells has been shown to be enhanced 4-5 times after exposure to low dose-rate γ-rays at 0.002 Gy/min when compared to irradiation at 1 Gy/min. No difference was observed in the response of AT cells to either low or high dose-rate irradiation (Cox 1982).

The absence of a reduction in sensitivity of AT cells following low dose-rate irradiation has also been observed by Paterson et al (1985). Following irradiation with a high linear energy transfer (LET) source, the relative biological effectiveness (RBE) for normal cell killing shows a LET-dependent increase with a maximum RBE value at around 100 KeV/μm, while for AT cells RBE is much less dependent on LET and the maximum RBE value was found to be much lower than that in normal cells (Cox 1982, Tobias et al 1984). These findings suggest that a genetic defect involved in the ability to recover from underlying DNA lesions may occur in AT.

Evidence supporting this notion has been obtained from investigations into the recovery from potentially lethal damage (PLD). PLD is operationally defined as the damage which is conditionally lethal to cells depending upon the conditions of post-irradiation incubation (Phillips and Tolmach 1966). Normal cells are capable of gradually recovering from PLD during several hours post-irradiation incubation under non-cycling conditions, i.e., in plateau phase cell growth conditions or balanced salts solution, showing increased clonogenic viability. The maximum PLD recovery in normal human cells was observed after 4 to 6 hours post-irradiation incubation, while in AT cells PLD recovery was completely deficient (Weichselbaum et al 1978, Cox et al 1981, Cox 1982, Arlett and Priestley 1984, Utsumi and Sasaki 1984). Since PLD recovery is thought to be a reflection of cellular repair of underlying damage in DNA (Frankenberg et al 1984), the lack of PLD repair in AT cells may indicate an inability to repair DNA damage of AT cells. Furthermore, a survival curve of AT cells exposed to ionizing radiation typically shows a lack of a shoulder region (Cox 1982, Tobias et al 1984) which is thought to be associated with accumulation and repair of sublethal damage (SLD) (Elkind 1977). Therefore AT cells are assumed to be also deficient in SLD repair (Tobias et al 1984).

Sensitivity to chemical agents

AT cells consistently exhibit a higher than normal sensitivity to some chemotherapeutic drugs, e.g., bleomycin (Taylor et al 1979, Edwards et al 1981, Cohen and Simpson 1982, Lehmann and Stevens 1979, Morris et al 1983) and neocarzinostatin (Babilon et al 1985, Cohen and Simpson 1983, Shiloh et al 1982). These drugs are termed radiomimetic agents since they react by splitting DNA via free-radical mechanism (Hatayama and Goldberg 1980, Takeshita et al 1981).

The sensitivity of AT cells to an array of chemical agents, which mainly modify base moieties in DNA, has been a subject of some controversy. Hoar and Sargent (1976) showed that five out of six AT strains are more sensitive to the alkylating agent methylmethane sulphonate (MMS). Paterson and Smith (1979) also found a hypersensitivity to MMS and N-methyl-N'-nitro-N'-nitrosoguanidine (MNNG) in most AT cell lines examined. In contrast, no abnormal sensitivity of AT to MMS and MNNG was demonstrated by other authors (Arlett 1977, Arlett et al 1982, Barfknecht and Little 1982). Scudiero (1980) found that six AT strains tested showed higher sensitivity to MNNG but not to MMS. Arlett et al (1982) showed a slight but consistent increased sensitivity to methyl nitrosourea (MNU) in AT cells although no abnormal sensitivity to other alkylating agents (MNNG and MMS) was found in the same AT cell lines examined. In the case of the ethylating agents, ethyl nitrosourea (ENU) and ethyl methane sulphonate (EMS) caused a higher lethality in AT than normal cells (Paterson and Smith 1979, Barkfnecht et al 1982), although Arlett et al (1982) reported no such increased sensitivity of AT cells to EMS and ENU. An increased sensitivity of AT cells to mitomycin C (MMC), which causes crosslinks in DNA, has been demonstrated by Hoar and Sargent (1976), while Arlett et al (1982) found that AT cells were similarly sensitive to MMC when compared to normal cells.

In addition, AT cells have been found to be more sensitive than normal cells to 4-nitroquinoline-1-oxide (4-NQO) (Barfknecht and Little 1982, Smith and Paterson 1980) and to some antibiotics, e.g., actinomycin D (Hoar and Sargent 1976), streptonigrin (Taylor et al 1985, Shiloh et al 1983a) and adriamycin as well as hydrogen peroxide (Shiloh et al 1983a). The cellular sensitivity of AT cells to a variety of DNA damaging agents is summarized in Table 1.1.

The spectrum of DNA damage induced by the agents to which AT cells are invariably sensitive suggests that the lesion may be DNA strand breakage (Shiloh et al 1985). Furthermore, agents such as streptonigrin, adriamycin and H_2O_2, together with bleomycin and neocarzinostatin, have the property in common with X-rays of producing intracellular free radicals. This fact has led Shiloh et al (1985) to suggest that AT cells are especially sensitive to a subgroup of DNA strand breaks induced via attack of free radicals that target the deoxyribose moiety of DNA.

Table 1. 1. Cellular sensitivity of AT cells to DNA damaging agents

Agents	Damage to DNA	Sensitivity
Radiation		
X- or γ-rays	1. strand breakage (broken at a phosphodiester bond or sugar moiety; or result from alkali-labile site or base damage) 2. relatively stable modifications in base or sugar moiety 3. crosslinks, etc.	H
α-particles	heavier and clustered damages as in the case of X-rays	H
UV (254 nm)	pyrimidine dimer	N
UV (310 nm)	non-dimer photolesions	H/N
Free radical-producing agents		
bleomycin	strand breakage (disassociation of phosphodiester bond, damage of sugar moiety, base release).	H
neocarzinostatin	strand breakage (disassociation of phosphodiester bond, damage of sugar moiety, base release).	H
Streptonigrin	strand breakage, bulk adduct	H
Base-modification agents		
MMS, MNNG, MNU	methylated adducts	H/N
EMS, EMU	ethylated adducts	H/N
Others		
MMC	crosslinks	H/N
4-NQO	strand breakage	H
Actinomycin D	intercalating into DNA	H

H: hypersensivity; N: normal sensitivity. H/N: reports for both hypersensitivity and normal sensitivity.

1. 1. 3. Cytogenetics

Spontaneous chromosomal aberrations

Chromosomal instability, both spontaneous or induced by ionizing radiation and radiomimetic agents such as bleomycin, is a universal characteristic of AT. Spontaneously occurring chromosomal abnormalities arise at higher frequencies in peripheral blood lymphocytes as well as in fibroblasts of AT than normal individuals (Hecht et al 1966, Oxford et al 1975, Cohen et al 1975, Taylor 1982). However, established lymphoblastoid cell lines are reported to show a normal level of spontaneous aberrations (Cohen et al 1979, Cohen and Simpson 1980). Spontaneous chromosomal aberrations include breaks, gaps and interchanges of both chromosome- and chromatid-type and symmetrical chromosomal rearrangements (translocations). In addition, telomeric dicentric chromosomes, formed by an end-to-end fusion of telomeres of two chromosomes, have been noted to occur at a high frequency in AT cells (Hayashi and Schmid 1975, Oxford et al 1975, Taylor et al 1981). The spontaneous frequency of sister chromatid exchanges (SCE) has been found to be normal in both lymphocytes of AT individuals (Galloway and Evans 1975, Hatcher et al 1976, Batram et al 1976, Hayashi and Schmid 1975) and AT lymphoblastoid cell lines (Cohen and Simpson 1982).

Comparing AT with other chromosome instability syndromes, e.g., Bloom's syndrome (BS) and Fanconi's anaemia (FA), there appears to be an obvious difference concerning the predominant type of spontaneous aberration: BS and FA individuals show a high level of spontaneous chromatid breaks and gaps and characteristic types of chromatid exchanges (symmetrical quadriradial formed from homologous chromosomes in the case of BS; triradial and quadriradial formed from non-homologous chromosomes in the case of FA) (Taylor 1982). In AT, however, although on

average chromosomal breaks are increased in comparison to normal cells (Gropp and Flatz 1967, Cohen et al 1975, 1979), there is evidence that they often overlap with the normal range (Taylor 1982). In contrast, the frequency of stable chromosomal rearrangements was found to be consistantly higher in all AT individuals (Kaiser-McCaw et al 1975, Aurias et al 1980, Oxford et al 1975, Taylor et al 1976, 1981, O'Connor et al 1982).

The distribution of chromosomal rearrangements in AT patients is apparantly highly non-random, always involving chromosome 7 and 14 and specially involving chromosome bands 7p14, 7q34, 14q12 and 14q32 (Aurias et al 1980, O'Connor et al 1982, Taylor 1982). The translocations observed include paracentric inversion of chromosome 14 and pericentric inversion of chromosome 7 and the most frequent translocations observed are t(7q;14q), and t(7p;14q) (Aurias et al 1980, O'Connor et al 1982, Taylor 1982). Using different mitogens to stimulate T- and B-lymphocytes, O'Connor et al (1982) observed a high frequency of rearrangements in AT cells at chromosome 7 and 14 in both groups of lymphocytes. Although rearrangements of chromosome 7 and 14 may also occur in normal individuals (Cohen and Simpson 1982, O'Connor et al 1982), the frequency was estimated to be 40-fold higher in AT cells than in normal cells (Taylor 1982). The breakpoint sites of rearrangement have been defined to involve T-cell receptor gene loci within chromosome 7 and 14 (O'Connor et al 1982, Taylor et al 1989). Furthermore, anomalies in chromosome 14 have shown a distinctive association with malignant neoplasms in AT patients (Keiser-McCaw and Hecht 1982).

Induced chromosomal aberrations

Frequencies of chromosomal aberrations induced by ionizing radiation and radiomimetic agents are dramatically increased in AT cells when compared to normal cells. Chromosomal hypersensitivity is generally

accepted as one of the hallmarks of the response of AT cells to ionizing radiation; two others are cellular hypersensitivity and radioresistant DNA synthesis. Higurashi and Conen (1973) firstly reported an elevated frequency of rings and dicentrics in γ-irradiated AT lymphocytes. When normal cells are irradiated at G_0 phase, only chromosome-type aberrations, namely rings, dicentris and fragments, are usually scored in the metaphse chromosomes. While irradiation of cells in the S- or G_2-phases results in chromatid-type aberrations, i.e., breaks, gaps and exchanges at the first mitosis. In lymphocytes from AT individuals, however, a pronounced increase in the number of chromatid-type aberrations appeared after G_0 irradiation and both the frequencies of chromatid breaks and gaps were observed to be more than 10-fold higher in AT cells when compared with normal cells following G_0 irradiation (Taylor et al 1976, Taylor 1978). In contrast to the increased frequency of chromosome fragments, rings and dicentrics occured at a normal level in AT cells particularly at higher doses (4 Gy) (Taylor et al 1976). It is notable that chromatid exchanges in these cases increased by a factor up to twenty in AT lymphocytes following G_0 irradiation (Taylor et al 1976, Taylor 1978). Natarajan and Meyer (1979) also failed to observe an increase in dicentric frequency in irradiated G_0 AT cells, however a 4-fold increase in chromatid aberrations in AT lymphocytes exposed to X-rays in G_0 was observed. The striking feature of the high frequency chromatid aberrations in AT cells irradiated in G_0 suggested a failure in the efficient repair of DNA damage in AT cells following ionizing radiation and that the unrepaired or misrepaired damage expresses itself as chromatid aberrations at the next mitosis (Taylor et al 1976, Taylor 1978).

AT cells irradiated at G_2 phase also show a higher frequency of chromatid aberrations when compared with normal G_2 cells (Rary et al 1974, Taylor et al 1976, Natarajan and Meyer 1979, Mozdorani and Bryant 1989a). Observations of Taylor (1982) suggest a ten- to twenty-fold increase in

chromatid breaks and gaps in AT lymphocytes irradiated in G_2 phase, whereas Bender et al (1985) reported that X-ray-induced chromatid deletions increased only 4-fold in AT lymphocytes exposed in G_2 phase. The frequency of chromatid exchanges is also reported to be higher in X-irradiated cells from AT patients (Taylor et al 1976, Taylor 1978). Higher yields of chromatid deletions can be observed in AT fibroblasts even at short times following X-irradiation (e.g., 1 hour including 30 min colcemid) (Mozdarani and Bryant 1989a). Following exposure to neutrons in G_2 phase, AT lymphocytes yielded about ten times more chromatid aberrations when compared to normal cells; the extent of increase being similar to that following X-irradiation (Natarajan et al 1982, Taylor 1982). Increased chromosomal sensitivity to bleomycin has also been demonstrated in AT cells (Taylor et al 1978).

It is possible to examine chromosomal damage in interphase cells by utilizing the technique of premature chromosome condensation (PCC). This technique visualizes chromosome fragments in interphase cells by the fusion of irradiated cells with unirradiated mitotic human or hamster cells in which the test chromosomes can be distinguished from those of the mitotic cells by 5-bromodeoxyuridine (BrdU) pre-labelling of the latter (Cornforth and Bedford 1983). The advantage of this technique is the possible determination of initial chromosome breaks induced by DNA damaging agents in all cells without the neccessity of cells reaching mitosis. The number of PCC fragments measured immediately after irradiation was found to increase linearly as a function of radiation dose (Cornforth and Bedford 1985, Pandita and Hittleman 1992). This observation is also true for cells treated with bleomycin (Hittleman and Sen 1988). Using the PCC technique Cornforth and Bedford (1985) found that the initial levels of chromosome breaks in X-irradiated G_1 phase AT fibroblasts were no different from that in G_1 normal cells. By contrast, using an alternative

technique for investigating chromosomal repair, namely G_2 assay which monitor the kinetics of chromatid aberrations in G_2 cells as a function of post-irradiation incubation time, the frequency of initial chromatid deletions was estimated to be appoximately 2.5-fold higher in X-irradiated AT fibroblasts than normal cells (Mozdarani and Bryant 1989a). A similar enhanced level of initial chromosomal damage in AT cells after exposure to ionizing radiation was found in the study of Pandita and Hittleman (1992) by measuring PCC in G_1 and G_2 cells. They reported a 2-fold higher initial chromosome breaks in G_1 and G_2 phases AT lymphoblastoid cell lines following γ-irradiation when compared with normal cells. An increased frequency of initial PCC breaks in G_1 phase AT fibroblasts after bleomycin treatment was also observed (Hittleman and Sen 1988).

Modification of chromosomal sensitivity

Natarajan et al (1980a) have studied the influence of post-treatment of caffeine on the level of X-ray induced chromosomal aberrations in blood lymphocytes from AT and normal individuals. Caffeine is known to release the radiation-induced G_2 arrest and to increase chromosomal aberrations in irradiated cells (Lücke-Huhle et al 1983). Post-irradiation treatment with caffeine potentiated the chromosome breaking effects of X-rays in AT to a similar extent as in normal cells (Natarajan et al 1980a). Cells in late S- or G_2 phases of both AT and normal cell lines have proved to be more sensitive to caffeine post-irradiation treatment than G_0 cells. This indicated no difference in the caffeine-sensitive lesions that were expressed as chromosomal aberrations in mitosis between AT and normal cells. Treatment of G_2 AT and normal human fibroblasts with 9-β-D-arabinofuranosyladenine (ara A), an inhibitor of DNA synthesis (reviewed by Cohen 1976), resulted in an increase of chromatid aberrations induced by irradiation to a similar extent between AT and normal cell lines (Mozdarani

and Bryant 1989a, b). Ara A did not increase the initial yields of chromosome deletions but did inhibit their repair in both AT and normal cells irradiated in G_2 phase (Mozdarani and Bryant 1989a, b). Inhibition of the repair of X-ray induced chromatid aberrations by 9-β-D-arabinofuranosylcytosine (ara C), which also inhibits DNA replication (reviewed by Cohen 1976), has also been observed in G_2 phase AT and normal cells, although unlike ara A, ara C additionally caused an increase in the frequency of chromatid deletions during the post-irradiation incubation (Mozdarani and Bryant 1988).

1. 1. 4. DNA Repair in AT cells

DNA damages induced by ionizing radiation

Ionizing radiation causes a number of different types of lesions in DNA, these are: single-strand breaks (ssb), double-strand breaks (dsb), base damage and crosslinks. Strand breaks can either arise from cleavage of the phosphodiester bonds or from disruption of a sugar moiety. Two ssb occuring opposite to one another or few bases apart in distance maybe regarded as a dsb. The majority of DNA breaks are ssb (approximately 1000 ssb/cell/Gy) compared with relatively low production of dsb (40 dsb/cell/Gy) (Taylor 1978, Blöcher and Pohlit 1982). Base damage involves alteration of base side groups or of the ring structure and most of the modifications have not been chemically characterized. The frequency of base damage is thought to be similar or even higher than the frequency of strand breaks. Crosslinks occur both between the strands of DNA and between DNA and protein. The frequency of DNA-protein crosslinks is estimated to be approximately 133 crosslinks/cell/Gy, and DNA-DNA crosslinks 30/cell/Gy. Of these lesions, the dsb is thought to be the lesion leading to cell death (Blöcher and Pohlit 1982, Bryant 1984).

Induction and rejoining of strand breaks

Measurements of DNA strand breaks in intact cells can be achieved by use of following methods: 1) classical analysis of a DNA profile with different molecular weights on an alkaline or a neutral sucrose gradient during velocity sedimentation (McGrath and Williams 1966, Lehmann and Stevens 1977); Dsb can be determined by neutral velocity sedimentation while ssb together with dsb determined by alkaline velocity sedimentation; 2) filter elution of DNA through a nucleopore filter in which the rate of elution is thought to depend on the molecular weight of DNA fragments (Kohn and Grimeg-Ewig 1973). This method can either be used under alkaline conditions for measurement of the combined frequency of ssb and dsb (Kohn and Grimeg-Ewig 1973) and crosslinks (Kohn et al 1980), or at pH 7.4 or 9.6 for measurement of dsb (Bradley and Kohn 1979). 3) DNA unwinding methods to determine the rate of unwinding from break points in the DNA helix under alkaline conditions (Ahnström and Erixon 1973). This method, like alkaline elution, measures a mixture of ssb and dsb. It can be used to examine dsb repair on the basis of different repair kinetics between ssb and dsb (Bryant and Blöcher et al 1980); 4) pulse field gel electrophoresis (PFGE) measures dsb by the migration of DNA double-strand fragments of varying molecular weight (Schwartz and Cantor 1984).

It has been found that enhanced radiosensitivities in some tumour cell lines are related to an increased induction of dsb by ionizing radiation in these cells (Peacock et al 1989). This is not true for AT cells since the induction of ssb and dsb caused by X- or γ-irradiation in AT cells is identical to those in normal cells (Lehmann and Stevens 1977, Coquerelle et al 1987, Peacock et al 1989). Irradiation of AT cells by α-particles also induces a similar frequency of dsb as in normal cells (Coquerelle et al 1987). The initial number of PCC has been found similar in AT and normal quiescent

fibroblasts (Cornforth and Bedford 1985), while a conflicting result reporting a higher production of initial PCC breaks in G1 as well as in G_2 phase was obtained in 3 AT lymphoblastoid cell lines (Pandita and Hittleman 1992).

A number of experiments have demonstrated normal rejoining of strand breaks in AT cells by utilising various techniques to determine ssb or dsb. Using alkaline sucrose gradient sedimentation, AT cells have been demonstrated to have a normal ability to rejoin ssb after irradiation (Taylor et al 1975, Vincent et al 1975, Paterson et al 1976). A normal rate and extent of ssb rejoining in AT cells has been confirmed by using procedures with higher sensitivity, e.g., alkaline unwinding (Sheridan and Huang 1979a, Thierry et al 1985) and alkaline filter elution (Fornace and Little 1980, Hariharan et al 1981). Sheridan and Huang (1979b) found no difference in the kinetics of rejoining of ssb between AT and normal cells following irradiation using an alternative approach in which following irradiation alkali-denatured DNA is treated with a single strand-specific endonuclease (S1 nuclease). The existence of ssb results in single-strand DNA after alkali denaturation which are digested by the nuclease so that the number of ssb induced by irradiation is inversely proportional to the remaining intact DNA.

The rejoining of dsb in AT cells has been found to be normal by neutral velocity sedimentation (Lehmann and Stevens 1977), or by neutral filter elution (Fornace and Little 1980, Van der Schans et al 1983, Thierry et al 1985). One exception to these observations was one AT cell line (AT2BE) which, using neutral filter elution, demonstrated that the early kinetics of dsb rejoining after irradiation were reduced in this AT strain, although the eventual extent of dsb rejoining was no different from that of normal cells (Coquerelle and Weibezahn 1981, Coquerelle et al 1987). The ability of AT cells to rejoin dsb induced by bleomycin (Fornace and Little 1980), neocarzinostatin (Shiloh et al 1983b), and 4-NQO (Van der Schans et al 1982)

was also found to be the same as that of normal cells. However, a higher level of residual dsb following 3 hours post-irradiation incubation was observed in AT2BE cells in comparison to normal cells after bleomycin treatment (Coquerelle et al 1987).

Although the rejoining of bulk ssb or dsb is normal in AT cells, a small number of breaks may remain unrepaired in AT cells after a lengthy incubation time. The number of residual breaks may be undetectable by the techniques developed so far. This was postulated by Taylor (1978) from results of chromosome studies and also by Lehmann and Stevens (1979). The thesis that more residual breaks are present in irradiated AT cells is particularly suggestive of deficient dsb rejoining, since the increased yields of chromosome aberrations found in AT cells following irradiation may arise from unrepaired dsb. With the PCC technique, the relationship between unrepaired dsb and chromosomal breaks has been investigated. X-ray induced PCC fragments were found to decrease during post-irradiation incubation and this was found to be mirror image of PLD repair as measured by a clonogenic assay (Cornforth and Bedford 1983). The half time for disappearance of PCC has been estimated at 2 hours (Cornforth and Bedford 1983), similar to the half time for dsb rejoining (Bryant and Blöcher 1980, Blöcher and Pohlit 1982), suggesting dsb as a possible origin of PCC breaks. Although PCC fragment rejoining shows normal kinetics in AT cells, the frequency of residual breaks was found to be 5 - 6 times higher in AT than in normal cells (Cornforth and Bedford 1985). Unlike measurements of bulk ssb or dsb, the doses of irradiation used in PCC technique are often within the dose range used for cell survival studies. The number of residual PCC fragments in AT cells after exposure to 6 Gy has been estimated at 10 breaks per cell compared to 2 breaks per normal cell (Cornforth and Bedford 1985). Only about 15% of initial dsb are immediately expressed as breaks in prematurely condensed G_1 chromosomes (Cornforth and Bedford 1983).

This indicates that appoximately 4% and 0.8% of initial dsb in AT and normal cells respectively, are probably not rejoined, and the difference of unrejoined dsb between AT and normal cells are too small to detect, particularly considering the errors in measurements.

Apart from unrepaired strand breaks, the mis-rejoined strand breaks play a large role in the determination of radiosensitivity. A number of experiments using recombinant DNA plasmids have been designed to investigate the fidelity of dsb rejoining in AT cells (see review of Thacker 1989). Dsb are generated by restriction endonucleases (RE) at a specific site in a selectable gene of plasmid and correct rejoining of dsb is identified by the restoration of the selectable gene function following transfection (Cox et al 1984, Debenham et al 1987, North et al 1990). Using the plasmid pSV2*gpt*, which is cut by RE at *gpt* coding or non-coding sites, to subsequently transform human fibroblasts, Cox et al (1984) found a markedly reduced transformation rate resulting from a failure of the correct rejoining of dsb in AT cells. The mis-repair of dsb in AT cells leads to large deletions and rearrangements around the site of scission on the plasmid (Cox et al 1986). In addition, Cox et al (1984) found that in normal cells the transformation of *Kpn* I-digested plasmid is dramatically reduced by treatment of the cut plasmid with S1 nuclease, but the transformation was much less affected by the enzyme treatment in AT cells. It was suggested that the reduced fidelity of rejoining of dsb may be a result of dis-equilibrium between ligation and exonuclease digestion of dsb in AT cells (Cox et al 1984, Debenham et al 1987). North et al (1990) examined the rejoining efficiency and fidelity of RE-induced dsb in plasmids incubated in nuclear extracts from human cells and found a higher frequency of mis-rejoining of plasmids when incubated with AT extracts compared with extracts from normal cells, although these extracts gave similar rejoin efficiencies. A higher proportion of incorrect rejoining of RE-cut plasmids in AT cells has also been demonstrated by

several other laboratories (Miyajima et al 1993, Powell et al 1993). It has been postulated that in AT nuclear extracts the broken ends of plasmids lack a normal protection by nuclear protein(s) (North et al 1990).

However, in spite of these observations showing a higher risk of incorrectly rejoined RE-induced dsb in AT cells, no such difference in repair fidelity for a shuttle plasmid was shown in γ-irradiated AT and normal lymphoblastoid cells (Sikpi et al 1992). It is known that ionizing radiation induces various types of DNA damage and there seems to be a specific lesion(s) which AT cells are not able to deal with correctly. The dsb is the most likely candidate for such a lesion.

Excision Repair

A variety of methods have been employed to investigate excision repair in mammalian cells. For example, a type of base damage, whose precise chemical nature is presently unknown, can be recognised and removed by an endonuclease present in *M. luteus* extract and results in single-strand breaks in DNA. These sites are termed endonuclease sensitive sites (ESS). Repair of this type of base damage can be determined by the disappearance ESS. Paterson et al (1976) detected that the removal of ESS in three out of four AT strains tested proved to be much slower than that in normal cells after ionizing irradiation. Further examination of repair synthesis, as measured by isopycnic centrifugation of DNA, containing both density and radioactive label, found eight out of thirteen AT fibroblast lines exhibited a lower level of repair replication induced by γ-irradiation (Paterson et al 1982). These findings led to the classification of AT cells into two groups of excision proficient (exr^+) and deficient (exr^-). The defect in excision repair in AT following ionizing radiation, however, has not been reproduced by other authors (Van der Schans et al 1980, Fornace et al 1986), using the exr^- AT cell lines reported by Paterson et al (1976, 1978). AT cells

show a temporary reduced level of unscheduled DNA synthesis (UDS) following γ-irradiation (Vincent et al 1980). Paterson et al (1982) observed that a reduced level of radiation-induced UDS occur in AT *exr*$^-$ strains, but not in AT *exr*$^+$ cells.

AT cells were found to have a consistently normal rate of repair synthesis after treatment with MMS (Lehmann and Stevens 1980, Scudiero 1980), while variable results were found after MNNG treatment. It was noted by Scudiero (1980) that defective repair synthesis was evident in four AT strains and that these strains also showed a cellular sensitivity to MNNG. However, Shiloh and Becker (1982) showed that the ability of six AT lymphoblastoid cell lines to remove MNNG-induced purine methylating adducts resembles non-AT cell lines, a result which is consistent with the normal clonogenic sensitivity to MNNG found in these AT strains (Shiloh et al 1985). Normal excision repair has been found in AT cells after treatment with MMC (Shiloh et al 1980) and streptonigrin (Taylor et al 1985), reflecting a normal excision of crosslink and of bulk adducts in DNA.

Repair enzymes

A number of enzymes which may be involved in DNA repair have been investigated in AT cells. DNA ligase I and II have been reported to be normal in AT cells (Willis and Lindahl 1987) as are polymerases (pol) α, β, and γ (Bertazzoni et al 1978), although contrary data has shown that the mean value of specific activity of pol β obtained from five AT fibroblast cell lines is 2-fold higher than the mean value of that from normal cells (Mitchell et al 1985). The levels of an enzyme, which removes one of the major types of base damage (5,6-dihydroxydihydrothymine), was found to be no different in AT and normal cells (Remsen and Cerutti 1977). A purified apurinic (AP) endonuclease from AT cells was shown to have a deficiency

in AP DNA binding activity (Kuhnlein 1985). Inoue et al (1977, 1982) have reported that AT cells have normal apurinic-site specific endonuclease, but are deficient in the action of a primer-activating-enzyme, which functions to modify the radiation-induced "dirty" 3' ends of breaks, allowing DNA polymerisation to subsequently fill the gaps. Deficiencies in the protection of broken ends of DNA in AT cells has been suggested to involve a deficient poly(ADP-ribose) polymerase (Miyajima et al 1993) which normally binds to dsb to protect the broken ends from exonucleolytic digestion (Satoh and Lindahl 1992). The level of activity of poly(ADP-ribose) polymerase has been found to be normal in unirradiated AT cells (Edwards and Taylor 1980, Zwelling et al 1983). Gamma-irradiation of normal lymphoblastoid cells resulted in an increased poly (ADP-ribose) synthesis as measured by ^{14}C-NAD incorporation, while this stimulating effect of irradiation on the activation of poly(ADP-ribose) was found to be deficient in two AT cell lines (Edwards and Taylor 1980). However, Zwelling et al (1983) observed a normal poly(ADP-ribose) synthesis in a number of fibroblasts and in two out of four AT lymphoblastoid cell lines following X-irradiation. With respect of topoisomerases, Topo I activity in AT cells was found to be the same as normal cells, whilst Topo II activity was found to be at least 10-fold reduced in 4 out of 5 AT cell lines (Singh et al 1988, Davies et al 1989, Lavin and Singh 1990). On the other hand, Topo II was observed to be abnormally overproduced in AT cells (Singh et al 1988, Singh and Lavin 1989, Smith and Makinson 1989).

Recombination repair

It has been proposed that the rejoining of dsb occurs through the mechanism of recombination. An excision-polymerization-ligation model can not account for dsb repair because a dsb has lost the template of the broken site (Resnick, 1976, Szostak et al 1983). On this model, a dsb can be

rejoined by utilizing the homologous sequence on an undamaged allele. Observations from the rejoining of RE-induced dsb in plasmid DNA suggests a proficient recombination in AT cells (Cox et al 1984). By sequencing the plasmids mis-rejoined by human nuclear extracts, Thacker et al (1992) found that mis-rejoined sites, either deletions or insertions, are exclusively between short direct repeats, which may be mediated by a sequence spliced recombination events. This finding implies that a frequent strand-exposure resulting from an exonuclease or helicase action followed by sequence splicing recombination occurs in AT cells (Thacker et al 1992). Powell et al (1993) demonstrated that undigested circular plasmid are also subjected to a high risk of deletion and insertion in addition to linearized plasmid DNA in AT cells, suggesting an error-prone recombination mechanism may be involved.

V(D)J recombination is a site-specific reaction that assembles variable (V), diversity (D), joining (J) segments of genes for immunoglobulin and T cell receptor during developments of these proteins in premature lymphoid cells. Rearrangements are mediated by a recombination signal sequence (RSS) that flanks all recombinationally competent V, D and J gene segments. It has been possible to activate V(D)J recombination in nonlymphoid cells by transfecting them with recombination-activating genes, RAG-1 and RAG-2 (Schatz et al 1989, Oettinger et al 1990). V(D)J recombination has been suggested to share some common components with DNA repair processes. A good example of this is the mouse severe combined immunodeficiency (*scid*) mutant (Bosma et al 1983). The X-ray sensitive *scid* mouse exhibits a defective V(D)J recombination as well as a defective repair of dsb (Schuler et al 1986, Biedermann et al 1991, Hendrickson et al 1991). A deficient V(D)J reaction has been identified in several other dsb repair-deficient hamster mutants (Pergola et al 1993, Taccioli et al 1993). Using a recombinant plasmid coding for RSS, it has been found that one or both components of the signal

or coding formation for the V(D)J recombination reaction is deficient in *xrs* 5, XR-1, V-3 and XR-V9B cell lines (Pergola et al 1993). These mutant cell lines represent four complementation groups of radiosensitive hamster mutant lines (Collins 1993). In contrast, proficient V(D)J recombination was found in two X-ray sensitive hamster mutant lines, V-E5 and V-G8 (Zdzienicka et al 1989) which show an AT-like phenotype, namely a higher radiosensitivity, radioresistant DNA synthesis and normal strand break repair (Pergola et al 1993). Recently, Hsieh et al (1993) reported that all aspects of V(D)J recombination are entirely normal in AT fibroblasts. Proficient V(D)J recombination is also found in cells from a Bloom's syndrome (BS) patient and a cell line (46BR*neo*) from an immunodeficiency disorder, indicating that immunodeficiency is not neccessarily related to defective V(D)J recombination. The fact that some BS lines and 46BR*neo* cells exhibit a deficient ligase I activity suggests that ligase I is unlikely to be required for the V(D)J reaction (Hsieh et al 1993). Therefore, the repair defect of AT cells involving a recombination mechanism, if it exists, seems not to involve V(D)J recombination.

1. 1. 5. Response of DNA synthesis to irradiation

A hallmark for almost all AT cells is that they lack a normal inhibition of DNA synthesis in response to ionizing radiation. It has been known for some time that ionizing radiation induces an inhibition of DNA synthesis in normal cells (Watanabe 1974, Waters et al 1975, Painter and Young 1975, de Wit et al 1981), which is thought to result from a blockage of replicon initiation at low doses and at higher doses a block in chain elongation of nascent DNA. It has been calculated that a single ssb in a replicon is able to stop the initiation of DNA replication within a group of replicons (Povirk and Painter 1976). DNA damage induced by UV

irradiation (Kaufmann et al 1980, Painter 1985a, Rosenstein 1984), bleomycin (Edwards et al 1981, Cramer and Painter 1981, Morris et al 1983), neocarzinostatin (Shiloh and Becker 1982, Cohen and Simpson 1983), and several DNA alkylating agents (Shiloh et al 1985, Lehmann 1982) are capable of inhibiting DNA synthesis by the same mechanisms. Camptothecin and amsacrine, inhibitors of Topo I and II respectively, which stabilize the DNA strand breaks of "cleavable complexes", have shown inhibitory effects on replicon initiation at low drug concentrations in normal cells (Kaufmann et al 1991). It is thought that the down-regulation of DNA replication by DNA damage is mediated by nuclear protein factors (Painter 1983, Lamb et al 1989), probably in a *trans*-acting manner (Lamb et al 1989). The biological significance of this slowing down of DNA synthesis following DNA damage has been suggested to increase the time for repair before replication can fix the lesions (Painter and Young 1980, Tolmach et al 1980).

In AT cells, however, DNA synthesis was not found to be depressed to the extent found in normal cells after X- or γ-irradiation (Houldsworth and Lavin 1980, Painter and Young 1980, de Wit et al 1981). It was proposed that in AT cells replicon initiation is completely resistant to DNA damage and chain elongation at higher doses inhibited to much lesser extent when compared to normal cells after exposure to X-radiation (Painter and Young 1980, Mohamed et al 1986). Reduced inhibition of DNA synthesis has been widely observed in AT cells after treatment with bleomycin (Edwards et al 1981, Cramer and Painter 1981, Morris et al 1983), neocarzinostatin (Shiloh and Becker 1982, Cohen and Simpson 1983), 4-NQO (Mirzayans and Paterson 1991) and a number of other agents to which AT cells are hypersensitive (Shiloh et al 1983a, 1985). In these cases, radioresistant DNA synthesis is coupled to the enhanced cellular sensitivity in AT cells to DNA damage induced by the agents. In addition, AT cells exhibit a normal inhibition of DNA synthesis following UV irradiation or alkylating agent treatment,

which is in common with a normal cellular sensitivity to UV and alkylating agents (de Wit et al 1981, Jaspers et al 1982).

Radioresistant DNA synthesis seems to be a dominant phenotype in AT cells. The radiosensitivity with respect to cell killing can be restored in AT x normal hybrid cells (Komatsu et al 1989) or in an AT cell line transfected with DNA from normal cells (Lehmann et al 1986), while the reduced inhibition of DNA synthesis remained in the hybrids and transfected AT cells. Further support for the dominance of the reduced DNA synthesis comes from the studies of Mohamed and Lavin (1986), where the introduction of nuclear extracts from AT cells into normal cells can cause an AT-like phenotype of radioresistent DNA synthesis. The factor conferring this resistance appears to be a protein with MW of 25 Kdal, although the biochemical function of the protein is unknown (Mohamed and Lavin 1986). Taylor et al (1987) reported that two cell lines from AT patients showed moderate radiosensitivities compared with other AT and normal strains. However, these two AT strains exhibited similar diminished inhibition of DNA synthesis to that found in more typically radiosensitive AT lines. One AT fibroblast cell line even shows a reduced inhibition of DNA synthesis and normal sensitivity of cell killing to 4-NQO treatment (Mirzayans and Paterson 1991). These results imply that there lack a correlation between the radiosensitivity and the radioresistance of DNA synthesis in AT cells.

1. 1. 6. Chromatin structure anomaly

DNA in the nucleus of mammalian cells is organized and packaged into higher order structure as illustrated in Figure 1. 1. The DNA is bound by histones and non-histone proteins to form the nucleosome structure which is further packaged by supercoiling into "thick" (30 nm) chromatin

fibres. Chromatin fibres are further highly organized in supercoiled loops which are attached to a supporting structure termed the nuclear matrix (as described in Alberts et al 1983). Several lines of evidence indicated that the loop organization may be of relevance to a number of biochemical reactions, e.g., DNA replication and transcription (see review in Mullenders et al 1987) and probably also to repair mechanisms (Mullenders et al 1987).

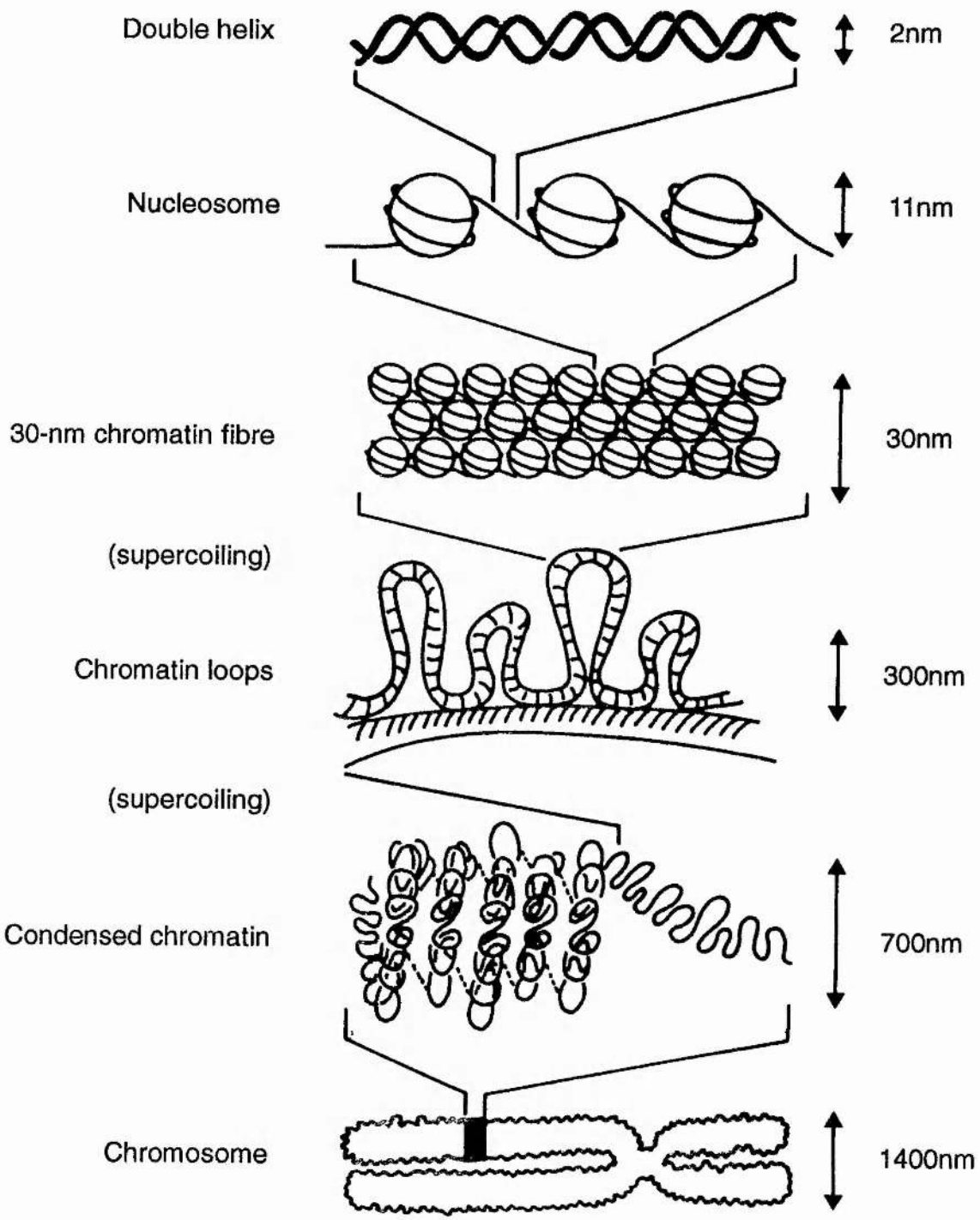

Figure 1. 1. Schematic illustration of chromatin organization and packaging of DNA from the double-helix to form the highly condensed metaphase chromosome. Redrawn from Alberts et al (1983).

Since there is no evidence to show a direct deficiency in DNA repair mechanisms in AT, a number of studies have investigated the possible anomaly of DNA structural organization within the AT cell nucleus, which may account for the abnormal responses of AT to ionizing radiation, such as cellular and chromosomal hypersensitivity and radioresistent DNA replication. Painter (1982, 1985b) suggested that the conformational alteration of DNA structure caused by radiation-induced DNA breaks may induce an inhibition of a number of replicons simultaneously by altering the binding of initiation complexes in normal cells. The inadequacy of such structural alteration therefore, could account for the abnormal response of DNA synthesis to radiation damage in AT cells (Painter 1985b). However, in unirradiated AT cells, no gross alteration in the supercoiled structure of DNA has been demonstrated (Lavin and Davidson 1981, Taylor et al 1991). Moreover, Lavin and Davidson (1981) investigated the nucleoid sedimentation characteristics in irradiated AT and normal cells. This assay measures restoration of DNA supercoiling structure as a result of rejoining breaks in nucleoid DNA. They found no difference in supercoiling restoration during post-irradiation incubation in irradiated AT cells compared to normal cells. Using an image analysis system, Taylor et al (1991) were able to directly visualize and measure the changes in nucleoid DNA loop size in irradiated cells. It had been noted that an increased extent of DNA loop unwinding is seen immediately after irradiation at 0 °C in two non-transformed AT lines compared with normal cells. This was thought to reflect a greater instability of the DNA nuclear matrix attachment points in irradiated AT cells (Taylor et al 1991). The ability of subsequent rewinding of DNA loops is apparently not deficient in AT cells (Taylor et al 1991), indicating a normal rejoining of DNA strand breaks which is consistant with the findings of Lavin and Davidson (1981). The abnormal extent of loop unwinding, however, has not been found in transformed

immortalized AT fibroblast and lymphoblast cell lines, although these cell lines exhibit higher sensitivity to ionizing radiation compared to normal cells (Taylor et al 1991).

The constitution and density of chromatin proteins plays an important role in protection of DNA against the induction and in subsequent repair of DNA damage. Evidence supporting an anomaly in condensation of AT chromatin is indicated by an enhanced susceptibility of DNA in AT cells to exogenous endonuclease (Smith 1984, Mohamed and Lavin 1989). However, in AT cells the major chromatin proteins, the histones show no difference from those in normal cells (Kraemer et al 1983). Mohamed and Lavin (1989) demonstrated a reduction in the DNA protein binding capacity of AT extracts. Since the difference in binding was obtained with lower salt concentration (0.35 mol/l) it is unlikely that abnormalities of histones or other major chromatin proteins will account for the change in AT cells. This is in agreement with data reported by Kraemer et al (1983). In addition, the reduced protein binding property of AT cells resulted in the access of restriction enzyme *Eco*R I to protein-bound plasmid when tested with plasmid pFF435B which contains the histone genes H2A and H2B and therefore possesses protein-binding capacity (Mohamed and Lavin 1989). Some studies have indicated abnormalities in the metabolism of some proteins which have a close association with the nuclear architecture (Murnance and Painter 1983, McKinnon and Burgoyne 1984). An overexpression of extracellular matrix proteins, fibronectin and collagen, and a number of matrix-degrating enzymes have been detected in AT fibroblasts (Murnance and Painter 1983, Aggeler and Murnance 1990) but not in AT lymphoblastoid cell lines (Lavin and Seymour 1984). Reduced levels of the cytoskeleton protein actin in AT cells have been observed (McKinnon and Burgoyne 1984). Subsequently, an altered array of actin-containing microfilaments appeared in AT cells (McKinnon and Burgoyne 1985).

Topo II is a major component with abundant binding sites throughout the nuclear matrix (Cockrill and Garrard 1986). It has been demonstrated that Topo II is involved in the maintainence of chromatin loop architecture during interphase and in chromosome condensation (Earnshaw et al 1985). The abnormal activity and expression of Topo II found in AT cells is therefore thought likely to relate to an alteration of chromatin structure (Singh et al 1988, Singh and Lavin 1989, Smith and Makinson 1989).

Recently, Almeida et al (1993) reported that genomic DNA, particularly in the repetitive sequence in lymphoblastoid cell lines derived from AT and Fanconi anemia (FA) patients are hypomethylated. They suggested a correlation between the hypomethylation of heterochromatic sequences in DNA and chromosome instability of the two chromosome instability syndromes. This result may also indicate an anomaly in the post-replication base-modification of DNA in AT and FA cells. It has long been suggested that DNA methylation, particularly of transcription control sequence, plays a regulatory role in gene expression during vertebrate embryonic development (see review of Tate and Bird 1993), whereas the biological significance of reduced methylation in unexpressed repetitive sequence is not clear.

1. 1. 7. Cell Cycle Perturbation

Irradiation of cells leads to an arrest in the progression of cells through the cell cycle such that cells accumulate in any stage of cell cycle (Smith et al 1985). The accumulation of cells is typically observed as a prolonged G_2 phase either for yeast (Weinert and Hartwell 1989) or mammalian cells (Smith et al 1985). This feature is thought to be a mechanism which allows cells to have a longer time to repair damage

before mitosis (Painter and Young 1980). Early studies of Zampetti-Bosseler and Scott (1981) showed by measuring the fraction of mitotic cells in the cell population that AT fibroblasts manifested a reduced radiogenic G_2 delay when compared with that of normal cells. However, several studies have reported that AT cells undergo a *longer* G_2 delay than normal cells when the kinetics of cell populations were determined using flow cytometric analysis (Imray and Kidson 1983, Ford et al 1984, Smith et al 1985, Bates et al 1985). The difference between these two sets of observations is possibly caused by the methods used (Ford et al 1984). It is now accepted that data from flow cytometric analysis reflects a whole population of cells that remain in G_2 phase following irradiation, while the measurement of the mitotic index does not include a portion of cells which died in interphase and are not subsequently capable of moving from G_2 into mitosis.

The kinetic data obtained by Smith et al (1985) suggest that the rates of accumulation of normal and AT cells into a G_2 delayed state are not significantly different, whereas a major difference is the absence of a later recovery in AT cells. Normal cells undergo a progressive accumulation in G_2 followed by a release from G_2 phase 24 hours after irradiation, while a high proportion of the AT cell population remain in G_2 without recovery. Studies of other cell cycle phases by flow cytometry show an abnormal cell cycle response throughout all phases in AT cells. For example, it has been shown that AT cells lack the G_1 delay present in normal cells (Nagasawa and Little 1983). Smith et al (1985) showed that irradiation causes a reduction in the G_1 population of both AT and normal cells, normal cells recover from the reduction earlier than AT cells. In normal cells, irradiation induces a transient accumulation of cells in S-phase, whereas S-phase accumulation is not found in AT cells (Smith et al 1985). The lack of arrest in S phase observed in AT cells shortly after irradiation appears to be correlated with their radioresistant DNA synthesis.

The nature of the lesion which leads to the altered cell cycle response following ionizing radiation is obscure. Caffeine is known to have miscellaneous effects on irradiated cells. It potentiates the cellular lethality of radiation, diminishes the inhibitory response of DNA synthesis in normal cells to DNA damage and reduces the G_2 delay in normal cells in responding to irradiation. Painter and Young (1980) has suggested that caffeine in some way binds to DNA and causes conformational changes in chromatin to cause AT-like effects (increased cellular sensitivity and reduced inhibition of DNA synthesis) in normal cells. In other words, AT cells may exist in a "caffeine-like" state (Painter and Young 1980). With respect to the effect on cell cycle, caffeine treatment reduces G_2 arrest of irradiated normal cells as well as of irradiated AT cells (Hansson et al 1984, Betes et al 1985). Similar results have been obtained by measuring mitotic indexes (Zampetti-Bosseler and Scott 1981). Therefore, the caffeine sensitive lesions seem to not relevant to those responsible for abnormal cell cycle response of AT to ionizing radiation.

In the yeast system, the G_2 delay after DNA damage was found to be controlled by a single gene, *rad9* (Weinert and Hartwell 1989). *RAD9* mutants lose the capacity that imposes G_2 arrest in response to DNA damage (Weinert and Hartwell 1989). In mammalian cells, the gene(s) which control the DNA damage-induced cell cycle delay remain unknown. The cell cycle checkpoint p53 protein in mammalian cells has proved to play a role in the arrest of cells in G_1 phase after DNA damage (Lane 1992). Accumulation of p53 occurs when DNA is damaged and this phenomenon has been proposed to consequently switch off replication to allow DNA to repair damage, therefore a lack of this response may increase genomic instability and sensitivity to DNA damage (Lane 1992). Evidence shows that ionizing radiation-induced p53 accumulation is defective in AT cells (Kastan et al 1992). In addition, Kastan et al (1992) found a deficiency in the induction of a

p53-associated gene in AT cells following irradiation, which indicates a possible defect in the binding property of p53 to this gene. In contrast to these findings, Lu and Lane (1993) have reported that the accumulation of p53 in response to ionizing radiation are not different between AT and normal cells.

1. 1. 8. AT Gene

AT is a recessive homozygous disorder and manifests itself when both AT genes of an allele of a chromosome are impaired. The AT gene has been suggested as a "housekeeping" gene (Gatti 1991). AT heterozygotes carry a normal and an abnormal AT gene and are clinically healthy. AT gene carriers occur at a high frequency of approximately 1/100 (Swift 1985). Evidence has shown that AT heterozygotes are at a higher risk of developing malignant neoplasms, in particular breast cancer (Peterson et al 1992). Therefore, predisposition of AT gene carriers is of importance. A large number of cell lines from AT obligate heterozygotes have been found to exhibit an intermediate sensitivity between that of AT homozygotes and that of normal individuals to ionizing radiation and various radiomimetic agents (Chen et al 1978, Arlett and Harcourt 1980, Paterson et al 1979, 1985, Shiloh et al 1982). Paterson et al (1985) have shown that the radiosensitivity of AT heterozygotes appears different depending on irradiation conditions; a higher than normal cellular sensitivity of AT heterozygous fibroblast lines to irradiation was observed under hypoxic conditions or at a reduced dose rate.

Four complementation groups for AT homozygotes have been identified based on chromosomal sensitivity (Chen et al 1984) and DNA replication capacity following irradiation (Jaspers et al 1988). These are disignated A (AB), C, D and E groups, respectively (Jaspers et al 1988). As

many as nine complementary groups has been predicted to exist in AT (Jaspers and Bootsma 1985). The AT genes for group A and C have been localised to chromosome 11q22-23 by linkage analysis (Gatti et al, 1988, Ziv et al 1991). The AT gene for group D has also been located to chromosome 11q22-23 by chromosome transfer and functional complementation studies (Lambert et al 1991). Taylor et al (1994) recently reported that AT gene for group E is localized on chromosome 11 at the same region as that for group A and C. Using a microcell-mediated chromosome transfer technique, normal copies of chromosome 11 have been transfered into AT cells and lead to a correction of the cellular phenotypes of AT, i.e., lead to an increased cell survival after streptonigrin treatment (Lambert et al 1991) and a decrease to normal in the frequency of chromosome aberrations after ionizing irradiation (Kodama et al 1992). These data provide strong evidence for defective gene(s) on chromosome 11 in AT cells. A candidate gene for AT group D (ATDC gene) has been cloned by Kapp et al (1992) by transfection of AT5BIVA cell line (complementation group D) with human cosmid library. They identified that ATDC gene contains overlapping DNA from chromosomal region 11q23 and is present in a single copy in the human genome. Southern (DNA) or Northern (RNA) blot analysis indicated no large rearrangement in ATDC gene in AT5BIVA cells, implying that any alteration in the gene in this cell line may involve a point mutation or a small rearrangement (Kapp et al 1992). The defective gene(s) for all the 4 complementation groups of AT seems to be localized in the same region of the long arm of chromosome 11, although AT gene for other unidentified rare groups may map outside this region (Hernandez et al 1993). This fact suggests that only a single wild-type AT gene may exsist with several functional domains, or that several genes are closely linked together.

1. 2. Specification of the biological role of double strand breaks by the use of restriction endonucleases

1. 2. 1. Double strand breaks are major lethal lesions induced by ionizing radiation

Exposure of cells to ionizing radiation induces various types of lesions in DNA including single- or double-strand breaks, base damage and crosslinks between DNA strands or between DNA and proteins. It is known that these lesions are not equally lethal to cells since cells possess an intrinsic ability to repair damage to DNA in order to maintain the integrity of genetic information. The mechanism of repair of DNA damage induced by ionizing radiation is far less clearly understood than that for UV-induced dimer lesions, which are repaired efficiently in cells through excision repair pathways (Freidberg 1984). Ionizing radiation-induced ssb, base damage and crosslinks are repaired by mechanisms which probably share some of the steps of excision repair (see review of Bryant 1989).

The repair of dsb is thought to be more complex than that of ssb and probably involves a recombination mechanism similar to that found in bacteria and yeast (Resnick 1976). The existence of recombination repair for dsb has been demonstrated using a cell-free system of mammalian cells (Moore et al 1986, Jessberger and Berg 1991). The kinetics of gross rejoining of dsb are found to be slower than those of the rejoining of ssb, possibly indicating that a more complex mechanism exists for dsb rejoining (Bryant and Blöcher 1980, Blöcher and Pohlit 1982). Increasing evidence has strongly suggested that dsb are crucial lesions responsible for the cellular, cytogenetic, mutagenetic and oncogenetic effects of ionizing radiation (see review of Bryant 1989 and Obe et al 1992).

It has been suggested that the increased cell killing effect of high LET radiation is due to the high production of dsb (Ritter et al 1977). A direct

relationship between dsb and cell lethality following irradiation has been established in bacteria and yeast systems by the utilization of dsb repair mutant strains (Hariharan and Hutchinson 1973, Bonura et al 1975, Ho 1975, Frankenberg et al 1981, 1984). One unrepaired dsb is capable of causing a lethal event in yeast *rad52* mutant which is deficient in dsb repair and recombination (Ho 1975, Frankenberg et al 1984). Blöcher and Pohlit (1982) proposed a one-to-one relationship between unrepaired dsb and cellular survival obtained after maximum repair of PLD in Ehrlich ascites tumour cells. By using radiation sensitive mutant cell lines, e.g., *xrs*, XR and *scid* mouse cell lines, there appears to be a striking linkage between a hypersensitivity to ionizing radiation and a deficiency in the ability to rejoin dsb (Kemp et al 1984, Giaccia et al 1985, Biedermann et al 1991). A defect in recombination processes in these mutant cell lines has been identified (Moore et al 1986, Pergola et al 1993, Taccioli et al 1993), which may indicate an association of dsb repair and recombination in mammalian cells.

Dsb are also thought to be the causative lesions which give rise to the chromosomal aberrations induced by ionizing radiation. An experiment performed by Natarajan and Obe (1978) provided evidence for the dsb origin of chromosomal aberrations. These authors introduced a single-strand specific endonuclease from *Neurospora*, which cleaves the strand opposite a ssb to form a dsb, into CHO cells permeablized with Sendai virus following X-irradiation. The conversion of ssb into dsb dramatically increased the frequency of all types of chromosomal aberrations in cells irradiated either at G_1 or G_2 phase, indicating a conversion of dsb rather than ssb into chromosomal aberrations (Natarajan and Obe 1978, Natarajan et al 1980b). Other useful data have been obtained from radiation sensitive mutant mammalian cell lines. Dsb repair-deficient Chinese hamster mutants (*xrs*) exhibited enhanced yields of chromosomal aberrations in response to ionizing radiation when compared with the CHO K1 parent line (Kemp and

Jeggo 1986, Bryant et al 1987, Darroudi and Natarajan 1987). Some radiosensitive mutant cell lines, e.g., *irs* (Jones et al 1987) and V-group mutants (V-C4, V-E5 and V-G8) (Zdzienicka et al 1989), show higher cellular and chromosomal sensitivities to ionizing radiation while showing a normal ability to rejoin dsb. Thus, incorrectly repaired dsb may also be responsible for cell inactivation and chromosomal aberrations.

The linkage between dsb and chromosomal aberrations has been reinforced by using restriction endonuclease in permeabilized living cells (Bryant 1984, Natarajan and Obe 1984). RE exclusively generate dsb in chromatin DNA and this allows the study of the biological consequences of dsb in the absence of other complicating lesions as would be produced in the case of irradiation. A large number of investigations using a wide variety of RE (Table 1. 2) have demonstrated that RE-induced dsb mimic ionizing radiation in causing cell death, chromosomal aberrations, mutagenesis and oncogenesis in mammalian cells (see review Bryant 1988).

Table 1. 2. Restriction endonucleases used in the literature.

RE	Recognizing sequence	Dsb end-structure	Cell lines used
Alu I	AG/CT	Blunt	CHO[8,14,16,18,20,26,27,32], V79[25, 28], XR-1[16], V-C4[25], Human lymphocytes[29], *xrs*-5[14], *xrs*-6[14]
Asu III	GPu/CGPyC	5'-2-base-overhang	CHO[23], PG 19 (mouse fibroblasts)[23]
*Bam*H I	G/GATCC	5'-4-base-overhang	CHO[6,12,17,23,34], V79[1,2,5,25], V-C4[25], V-G8[25], *xrs*-5[3]
Ban I	G/G(C,T)(A,G)CC	5'-4-base-overhang	AT5BIVA[13], MRC5V1[13], GM8505[13], 46BR1G1[13]
*Bst*N I	CC/A(T)GG	5'-2-base-overhang	CHO[27]
Bsp	G(G,A,T)GC(C,A,T)/C	3'-4-base-overhang	CHO[27]
Cfo I	GCG/C	3'-2-base-overhang	CHO[14,24], *xrs*-5[14], *xrs*-6[14]
Dra I	TTT/AAA	Blunt	CHO[11,30]
*Eco*R I	G/AATTC	5'-4-base-overhang	CHO[3,6,10,14,19,22,31,33,34], EM9[10], *xrs*-5[3,14], *xrs*-6[14]
*Eco*R V	GAT/ATC	Blunt	CHO[3,23], PG19[23]
Hae II	PuGCGC/Py	3'-4-base-overhang	CHO[24]
Hae III	GG/CC	Blunt	CHO[11,14,24,27], *xrs*-5[14], *xrs*-6[14]
Hpa I	GTT/AAC	Blunt	CHO[27]
Hpa II	C/CGG	5'-2-base-overhang	CHO[14,27], *xrs*-5[14], *xrs*-6[14]
Msp I	C/CGG	5'-2-base-overhang	CHO[27,32]
Nun II	GG/CGCC	5'-2-base-overhang	CHO[23], PG 19[23]
Pst I	CTGCA/G	3'-4-base-overhang	CHO[20,34]
Pvu II	CAG/CTG	Blunt	CHO[3,4,6,12,20,21,23,31], C3H101/2[7], V79[1,2,5,], *xrs*-5[3], *irs*-2[5], PG19[23], AT5BIVA[13], MRC5V1[13], GM8505[13], 46BR1G1[13]

(to be continued in next page)

Table 1. 2 (continue)

RE	Recognizing sequence	Dsb end-structure	Cell lines used
Rsa I	GT/AC	Blunt	CHO[27,32], *scid*[8], CB-17(mouse fibroblasts)[8]
Sac I	GAGCT/C	3'-4-base-overhang	CHO[27]
Sal I	G/TCGAC	5'-4-base-overhang	CHO[34]
*Sau*3A I	/GATC	5'-4-base-overhang	CHO[9,16,21,32], XR-1[16], *scid*[8], CB-17[8],
Sca I	AGT/ACT	Blunt	CHO[11]
Sma I	CCC/GGG	Blunt	CHO[23,24], PG19[23]
Taq I	T/CGA	5'-2-base-overhang	CHO[23]
Xba I	T/CTAGA	5'-4-base-overhang	CHO[20]

1. Bryant (1984); 2. Bryant (1985); 3. Bryant et al (1987); 4. Bryant (1992); 5. Bryant et al (1992); 6. Bryant and Christie (1989); 7. Bryant and Riches (1989); 8. Chang et al (1993); 9. Chung et al (1992); 10. Cortés and Ortiz (1991); 11. Cortés and Ortiz (1992); 12. Costa and Bryant (1991); 13. Costa and Thacker (1993); 14. Darroudi and Natarajan (1989); 15. Durante et al (1991); 16. Giaccia et al (1990); 17. Gustavino et al (1986); 18. Johannes and Obe (1992); 19. Morgan et al (1988); 20. Morgan et al (1990); 21. Morgan et al (1991); 22. Moses et al (1990); 23. Natarajan and Obe (1984); 24. Natarajan et al (1985); 25. Natarajan et al (1992); 26. Obe and Natarajan (1984); 27. Obe et al (1985); 28. Obe et al (1986b); 29. Obe et al (1986a); 30. Obe et al (1987); 31. Singh and Bryant (1991); 32. Winegar and Preston (1988); 33. Winegar et al (1989); 34. Zhang and Dong (1987).

1. 2. 2. Introduction of RE into mammalian cells

Early experiments by Bryant (1984) and by Natarajan and Obe (1984) used inactivated Sendai virus to permeabilize cells. The virus produces pores of approximately 1 nm diameter in cell membranes (see review Bryant 1992). Other methods applied involve a hypertonic exposure of cells to various agents in the presence of RE, e.g., glycerol, sorbitol (Obe et al 1985, Johannes and Obe 1991) or polyethylene glycol (Winegar and Preston 1988).

These methods involve an endocytosis mechanism that forms pinocytotic vesicles containing RE in the cytoplasmic membranes during the osmotic shock, and which releases RE from vesicles inside the cells after hypertonic treatment (Obe and Winkel 1985, Johannes et al 1992).

RE can be produced inside mammalian cells by the expression of the enzyme's gene inserted in a recombinant plasmid. In CHO cells the expression of the *Eco*R I gene, which is controlled by the mouse metallothionein gene promoter and regulated by the presence or absence of heavy metal ion such as Cd^{++}, has been achieved (Morgan et al 1988, Winegar et al 1989). The advantage of this method is that a high frequency of RE damaged (90%) transformed cells can be selected by *neo* resistance from the *neo* gene also encoded by the plasmid.

One of the most widely used methods for introducing RE into cells is electroporation (Wineger et al 1989, Costa and Bryant 1990a, Moses et al 1990). During a high voltage discharge, electroporation generates pores of 2 - 4 nm in cell membranes which allow RE to penetrate the cytoplasm. Electroporation has been demonstrated to be a more efficient method of permeabilization of cell membranes in comparison to the methods which employ hypertonic shock (Wineger et al 1989, Johannes and Obe 1991). However, electroporation causes the lysis of a large proportion of the electroporated cells and only approximately 30% of the electroporated cells are reported to remain viable (Lambert et al 1990, Bryant 1992).

A new method for cell poration has been developed recently by the utilization of a bacterial cytolysin, streptolysin-O (SLO) (Bryant 1992). SLO is a protein with a MW of 69 Kdal produced by bacterial strains of *Streptococcus pyogenes*. SLO targets cholesterol to produce large primary lesions in cell membranes (Duncan and Schlegel 1975, Bhakdi et al 1985), which function as channels through which large molecules may passively pass. The pore size produced by SLO can be greater than 12 nm at higher

concentrations, allowing the release of radio-labelled proteins with a MW of up to 483 Kdal from the cytoplasm of loaded red cell ghosts (Buckingham and Duncan 1983). SLO poration has shown to be of benefit in facilitating the uptake of macromolecules by cells and increasing the proportion of live porated cells which are able to progress through the cell cycle for subsequent cytogenetic examination (Bryant 1992).

1. 2. 3. RE-induced dsb mimic radiation effects on cells

RE were first used to mimic the cytogenetic effects of ionizing radiation in Chinese hamster V79 cells (Bryant 1984). This work showed that treatment of permeabilized V79 cells with *Pvu* II leads to chromosomal aberrations, of both the deletion and exchange types, similar to those observed in cells exposed to ionizing radiation. Bryant (1984) also determined a RE dose-dependent induction of dsb in RE-treated cells, showing that the aberrations do indeed originate from dsb in DNA. Natarajan and Obe (1984) concomitantly found that RE-induced dsb give rise to chromosomal aberrations in CHO cells and mouse fibroblasts in G_1 or G_2 phase of the cell cycle. In addition, the chromosome-breaking effect of RE has been reported in human peripheral lymphocytes (Obe et al 1986a). The types of chromosomal aberrations induced by RE are essentially the same as those induced by radiation, i.e., mainly chromatid-type aberrations in late S or G_2 phases and mainly chromosome-type aberrations in G_1 and early S phases cells (Obe and Winkel 1985). However, some studies show that the chromosome- and chromatid-type aberrations are present at the same time and even in the same metaphase after treatment of cells with RE in G_1 phase (Natarajan and Obe 1984). This phenomenon differs from that observed in G_1 phase cells exposed to ionizing radiation and has been explained by the hypothesis that RE are active in cells for a long period of

time after treatment (Natarajan and Obe 1984). RE were found to produce few chromosome aberrations in synchronized mitotic cells although there are indications that the DNA cleavage by RE occurred (Morgan et al 1991). This truly mimics irradiation in that no aberrations were found immediately after the irradiation of mitotic cells (Hsu et al 1962, MacLeod et al 1992). One possible explanation of this observation is that chromosome decondensation, DNA replication and chromosomal recondensation are required before aberrations are manifested. In addition to causing chromosomal aberrations, RE treatment was also shown to induce micronuclei in CHO cells (Moses and Bryant 1989, Bryant 1992).

Treatment of cells with RE has been shown to induce a higher level of SCE than is observed in control cells (Natarajan et al 1985, Stoilov et al 1986, Folle et al 1992). In contrast, Morgan et al (1988, 1989) showed no increase in SCE in CHO cells treated with RE. This difference may be the result of the cell culture state, e.g., at different stage of cell cycle, since expression of SCE has been found to be strictly cell-cycle dependent (Perry and Evans 1971, Folle et al 1992).

Like ionizing radiation, RE were found to cause mutations at the *hprt* locus which involve large genetic changes such as deletions, and the mutations were found to be associated with chromosomal aberrations (Obe et al 1986b). Higher than control rates of mutation at the *tk* locus have also been demonstrated following electroporation of *Pvu* II and *Eco*R I into CHO cells (Singh and Bryant 1991). No mutations at the Na^+/K^+ ATPase locus were found following treatment with RE or ionizing irradiation (Obe et al 1986b, Thacker et al 1978). This implies a connection between the nature of the pre-mutation lesions induced by ionizing radiation and those induced by RE. It has been suggested that major structural changes at the Na^+/K^+ ATPase locus may cause an inactivation of the gene and consequentially lead to cell death, while a base point-mutation e.g., induced by EMS, may not

completely inactivate the gene hence mutant cells are observed (Thacker et al 1978). Thus, a strong correlation exists between mutations induced by RE and those induced by ionizing radiation.

In addition, studies on oncogenic transformation have shown that *Pvu* II treatment induces an increased frequency of transformation foci in murine C3H10T1/2 cells (Bryant and Riches 1989, Borek et al 1991), a result similar to that found in the cell line after X-irradiation (Reznikoff et al 1973, Han and Elkind 1979).

Taking these findings together, there appear to be striking similarities in a number of end-points induced by ionizing radiation and those induced by RE, implying that RE-induced dsb lead to chromosomal aberrations and other biological consequences by a similar mechanism to those induced by ionizing radiation.

1. 2. 4. Cytogenetic effects of RE-induced dsb with different end-structure

RE recognize and incise specific sequence in the DNA double strand helix to produce dsb. The termini of dsb generated by RE are always 5'-phosphoryl and 3'-hydroxyl groups or so called "clean" ends since they may be readily rejoined by DNA ligase. In contrast, the dsb induced by ionizing radiation are thought to possess highly variable "dirty" termini which would require "cleaning" prior to ligation. RE induced dsb, in this sense, may not be identical to those induced by ionizing radiation.

However, RE-caused dsb mimic dsb induced by ionizing radiation in that they have blunt- or cohesive-termini. Dsb induced by ionizing radiation are either the result of a single event of radical attack through both strands of the DNA, forming a blunt-ended dsb, or the production of two closely generated ssb, forming a dsb with staggered end in either the 5′- or 3′-direction. The latter type of dsb, with variable degrees of base overlap, has

been assumed to be predominant among the dsb induced by irradiation since the probability of breaking the DNA at two sites exactly opposite one another in a single event would be very low (Bryant 1989).

It was suggested that the end-structure of RE-induced dsb are important in determining clastogenetic effects. Evidence has indicated that dsb with blunt-termini are more effective than dsb with cohesive-termini at inducing chromosomal aberrations. Bryant (1984) found that *Pvu* II which causes blunt-ended dsb is much more effective in inducing chromosomal aberrations in comparison to *Bam*H I which causes cohesive-ended dsb in virus permeabilized V79 cells although the production of dsb by both enzymes is the same when measured by the DNA unwinding method. This observation was confirmed by the work of Natarajan and his colleagues. They demonstrated that several RE causing cohesive-ended dsb show a reduced clastogenetic effect on CHO cells when compared to RE which induce blunt-ended dsb (Natarajan et al 1984, Darroudi and Natarajan 1989). In the study of Morgen et al (1990), *Pvu* II was found to be the most effective in the induction of chromosomal aberrations in CHO cells, in comparison to *Pst* I and *Xba* I, both of which induce dsb with sticky ends, in spite of the fact that out of these enzymes the lowest yield of dsb was produced by *Pvu* II as detected by pulsed-field gel eletrophoresis (PFGE). Using electroporation to introduce RE into cells, Moses et al (1990) found that blunt-ended dsb caused by *Pvu* II are more effective in the production of micronuclei and chromosomal aberrations in CHO cells when compared with cohesive-ended dsb produced by *Eco*R I. Evidence from cell survival experiments show that the RE producing cohesive-ended dsb also cause less cell death than the RE producing blunt-ended dsb (Bryant 1985, Giaccia et al 1990). Furthermore, a greater mutagenic effect was produced in the *tk* gene by *Pvu* II in comparison to that of *Eco*R I (Singh and Bryant 1991) in spite of the

presence of more restriction sites for *Eco*R I than for *Pvu* II within the *tk* gene.

Several other observations, however, have shown that under certain conditions, cohesive-ended dsb can be as efficient as blunt-ended dsb in the production of chromosomal aberrations (Obe et al 1985, Winegar and Preston 1988, Gustavino et al 1986). Obe et al (1985) found that treatment of cell "pellet" with RE inducing cohesive-ended dsb leads to chromosomal aberrations apparently as efficiently as those inducing blunt-ended dsb. Gustavino et al (1986) also reported that *Bam*H I causes a higher frequency of chromosomal aberrations in CHO cells in comparison with the data for *Bam*H I obtained by other authors (Bryant 1984, Natarajan and Obe 1984). Winegar and Preston (1988) showed that although *Sau*3A I is less effective in inducing chromosomal aberrations than *Alu* I, it produced more aberrations than another blunt-end producing enzyme *Rsa* I, and thus they argued that the cutting frequency rather than end-structure is more important in determining the clastogenicity of RE. The reason for these conflicting findings is not clear but has been addressed by Bryant and Christie (1989) in that it may be due to the methods used to permeabilise and treat cells and in some cases the use of very high RE concentrations, e.g., 12,500 units/ml (Obe et al 1985). They observed that no aberrations were induced by RE either generating cohesive- or blunt-ended dsb in the absence of glycerol (RE was purified to remove glycerol in the storage buffer), while aberrations appeared following addition of Sendai virus. Under the latter conditions, blunt-ended dsb were again found to be more effective than cohesive-ended dsb in the induction of chromosomal aberrations.

Zhang and Dong (1987) investigated a number of RE causing cohesive dsb in either the 3'- or 5'-direction in DNA and found that when they applied the "pellet" method, all of these enzymes (50 - 100 units) induce chromosomal aberrations in CHO cells. *Pst* I, an enzyme producing 3'-

overhang cohesive dsb, seems to produce a higher frequency of aberrations than other enzymes producing 5'-overhang aberrations. These findings suggest that RE-induced dsb with different end-structure may be repaired by different mechanisms.

1. 2. 5. Repair of RE-induced dsb

The induction of dsb by RE was found to follow a RE dose-dependent relationship (Bryant 1984). Comparison of RE-induced dsb in Sendai virus treated cells and X-irradiation induced dsb showed qualitatively that 500 units of *Pvu* II appeared to induce the equivalent frequency of dsb to that induced by 2 Gy X-irradiation in V79 cells when dsb were measured using the DNA unwinding technique (Bryant 1984, Bryant 1988). A similar observation was obtained by employing a nucleoid sedimentation method which showed that the production of dsb by 60-120 units of *Pvu* II is equivalent to that induced by 0.25 Gy of X-irradiation in a CHO cell line (Natarajan et al 1985). On this basis, Bryant (1988) calculated that 100 units of *Pvu* II induce on average of approximately 400 dsb per cell.

The repair of RE-induced dsb is difficult to investigate because, unlike the transient effect of irradiation, RE are thought to continuously generate dsb in DNA over a prolonged period and the processes of cutting and rejoining are thought to occur concomitantly (Costa and Bryant 1990a). Costa and Bryant (1990a) studied the kinetics of accumulation of dsb in electroporated cells using neutral filter elution. They showed that the frequency of dsb induced by *Pvu* II increase gradually with time of incubation over a period of hours, even after 24 hours. This indicates that RE persist and remain active for a considerable length of time inside cells and dsb appear as a result of the rate of incision of exceeding to the rate of rejoining of dsb.

The accumulation of dsb with blunt- or cohesive-ends appears to occur at a different rate. Using neutral filter elution, *Bam*H I-induced dsb with cohesive-ends were found to accumulate to a lesser extent than *Pvu* II-induced blunt-ended dsb, probably reflecting a higher rejoining rate for the former type of dsb (Costa and Bryant 1991a). The accumulation of dsb induced by *Sau*3A I has also been shown to be lower than those induced by *Alu* I when measured by PFGE (Giaccia et al 1990, Chung et al 1991). These results suggested that a different repair pathway for dsb with staggered ends and those with blunt-ends exists and it is reasonable to speculate that staggered ends are more rapidly rejoined.

As has been mentioned previously, dsb generated by RE are not chemically identical to radiation-induced dsb. Ionizing radiation-induced dsb with "dirty" ends requiring modification by exonuclease before further repair synthesis and ligation, while dsb with "clean" ends do not need exonuclease modification prior to ligation. The repair of RE-induced dsb, however, may be more complex than just a single ligation step. This is indicated by using DNA repair inhibitors, where it is possible to investigate the processing of RE-induced dsb in cells.

Natarajan and Obe (1984) observed that ara C, an inhibitor of DNA polymerization, increased the frequency of chromosomal aberrations induced by *Pvu* II and *Eco*R V. This has been interpreted to be due to the inhibition of DNA synthesis or ligation. The potentiation by ara C of chromosomal aberrations induced by *Alu* I was also demonstrated by Obe and Natarajan (1985). Caffeine is known to abolish the G_2 block induced by DNA damage in cells, and has been found to increase the frequency of *Pvu* II-induced aberrations significantly when applied to cells 2 hours before fixation. This indicates that RE-induced dsb share a caffeine-sensitive repair pathway with radiation-induced damage (Natarajan and Obe 1984).

Chung et al (1991) examined the potentiation effects of several DNA repair inhibitors, such as caffeine, ara C, aphidicolin and 3-aminobenzamide (3AB), an inhibitor of poly(ADP-ribosyl)polymerase, on RE-induced chromosomal aberrations. 3AB and ara C were found to potentiate chromosomal aberrations induced by both *Alu* I and *Sau*3A I, while caffeine had no effect on aberration yields induced by either enzymes. Aphidicolin was found to increase the level of chromosomal aberrations induced by *Sau*3A I but have no effect on those induced by *Alu* I. Dsb production measured by PFGE showed that no significant difference in the amount of dsb produced by the treatment with any of these agents, with the exception of 3AB, which showed a transient effect in increasing the number of dsb induced by both *Alu* I and *Sau*3A I. These data suggest that poly(ADP-ribosyl)polymerase and polymerase α and δ are important for the cellular processes required for the normal repair of dsb induced by RE.

1. 2. 6. Factors which influence RE activity in cells

To date very little is known about the entry and action of RE inside cells. It is reasonable to assume that the amount of different RE entering cells will not differ greatly for a particular cell line when using a certain method for cell poration, although it may differ between different cell lines which also appear to be differently sensitive to RE treatment. In addition, the cytogenetic effects of RE may be largely influenced by the tolerance of activity of RE under cellular conditions and the accessibility of RE to chromatin DNA.

Although the activity and stability of RE in cells is difficult to determine, attempts have been made to investigate the accessibility of RE to chromatin DNA. Treatment of cells with a high concentration (3.2 mol/l) of $(NH_4)_2SO_4$ followed by RE treatment leads to higher level of aberrations

than is observed when cells are treated with *Alu* I alone (Obe and Kamra 1986). This is explained by a structural alteration of chromatin resulting from the high salt concentration which leads to more recognition sites being recognizable and incised by the enzyme (Obe and Kamra 1986). It has been estimated that only 60% of the recognition sites for *Eco*R I are accessible to enzyme digestion in isolated calf thymus nuclei mainly because of chromatin condensation (Lipchitz and Axel 1976). These results indicate that the availability of DNA in chromatin to cleavage by RE depends on the organization of chromatin in the nucleus.

The frequency of recognition sites in human DNA for different RE vary due to a presumed non-random distribution of bases (Bishop et al 1983). It has been proposed that the cutting frequency is important in determining the efficiency of aberration induction by RE (Winegar et al 1988). In addition, some restriction enzymes are sensitive to base methylation and their activities would be prevented by this modification which frequently occurs in DNA. This has been demonstrated by using two isoschizomeres *Msp* I and *Hpa* II in which *Hpa* II but not *Msp* I is sensitive to methylation on cytosine residues (Obe et al 1985, Winegar et al 1990). In these studies a lower efficiency in the induction of chromosome aberrations was found for *Hpa* II treatment compared with *Msp* I. Similarly, base-substitution with BrdU in chromatin was found to prevent cleavage by RE recognizing thymine-rich DNA sequences (*Eco*R I, *Sca* I and *Dra* I) and subsequently lead to a suppression of the clastogenic activity of these enzymes, while the effect of *Hpa* II which does not have thymine in its recognition sequence is not influenced (Obe et al 1987, Cortés and Ortiz 1992).

1. 2. 7. Response of mutant radiosensitive cell lines to RE

A number of radiation sensitive rodent mutant cell lines have been investigated for their sensitivity to RE-induced dsb. Bryant et al (1987) investigated the sensitivity of X-ray-sensitive mutant *xrs*-5 line to RE-induced dsb. *Xrs*-5 cells yielded a higher frequency of chromosomal aberrations than its parental CHO K1 line after treatment with *Pvu* II and *Eco*R V (inducing blunt-ended dsb) and *Bam*H I and *Eco*R I (inducing cohesive-ended dsb) (Bryant et al 1987). Darroudi and Natarajan (1989) examined *xrs*-5 and *xrs*-6 cell lines and showed these mutant cell lines are 1.5 to 2-fold more sensitive to either blunt- (induced by *Hae* III and *Alu* I) or cohesive-ended dsb (caused by *Cfo* I, *Eco*R I and *Hpa* II) with respect to chromosomal aberrations, in comparison to CHO cells. The hypersensitivity of *xrs* lines to RE-induced dsb is thought to relate to the deficient dsb repair found in the mutants. A defect in the repair of RE-induced dsb in *xrs*-5 cells has been emphasised by the results obtained by Costa and Bryant (1991b) who showed that *xrs*-5 cells accumulate higher levels of dsb after *Pvu* II and *Bam*H I treatment compared with CHO cells.

Cortés and Ortiz (1991) found that *Eco*R I caused increased yields of chromosomal aberrations in EM9 cells when compared to its parental CHO AA8 line. The EM9 mutant was isolated for its hypersensitivity to EMS and it exhibits a cross-sensitivity to ionizing irradiation accompanied by a defect in the rejoining of DNA single strand-breaks (Thompson et al 1982), while dsb rejoining in EM9 cells has been reported to be essentially normal (VanAnkeren et al 1988). Although in this experiment, *Eco*R I was introduced into AA8 by electroporation and into EM9 cells by incubation with RE after trypsinizing, since electroporation resulted in a severe cell killing in the mutant cells, EM9 cells still showed about a 3-fold higher frequency of chromosomal aberrations when compared with the

electroporated parental cells (Cortés and Ortiz 1991). Another dsb repair deficient mutant cell line, XR-1, has been reported to show an enhanced clonogenic sensitivity to treatment with *Alu* I and *Sau*3A I compared with CHO cells, although the yield of dsb induced by the enzymes showed no difference between the mutant and the wild type lines (Giaccia et al 1990).

Cells from the autosomal recessive *scid* mice are similar to the Chinese hamster mutant cell lines mentioned above, displaying an enhanced radiosensitivity and defective rejoining of dsb which may be a result of a genetic defect involving a deficient V(D)J recombination. *Scid* cells have been shown to present in different complementation group from AT and lack the AT-like radiation-resistant DNA synthesis (Komutsu et al 1993). Chang et al (1993) demonstrated that *scid* cells exhibit increased cytotoxic response to RE. Electroporation of *Rsa* I (causing blunt-ended dsb) and *Sau*3A I (causing 5'-cohesive-ended dsb) into *scid* and the wild type CB-17 mouse cells, although inducing similar yields of dsb in both cell lines by either RE, led to 3- and 4-fold reduction of the clonogenic capability, respectively, in *scid* cells compared with CB-17 cells (Chang et al 1993).

A radiosensitive Chinese hamster mutant cell line, *irs*-2, resembles ataxia telangiectasia in many respects including a radiation resistant DNA synthesis and a normal ability to rejoin ssb and dsb. When *Pvu* II was porated by SLO treatment into *irs*-2 cells and wild type V79 cells, an enhanced level of chromosomal aberrations was found in *irs*-2 cells regardless of the fact that a similar amount of dsb were induced by *Pvu* II treatment in the two cell lines (Bryant et al 1993). An increased frequency of chromosomal aberrations appeared in *irs*-2 cells at fixation times of either 4 or 18 hours by factors of 3.7 and 2 - 3, respectively, indicating that *irs*-2 cells at both G_1 and G_2 phases are more sensitive to RE treatment when compared with V79 cells (Bryant et al 1993). This finding has increased our knowledge of the underlying mechanism by which chromosomal aberrations are

produced and it is becoming clear that it is not directly related to the ability of cells to rejoin dsb. A defect in processing of dsb induced by RE in *irs*-2 cells which subsequently leads to conversion of more dsb into chromosomal aberrations has been proposed (Bryant et al 1993).

The enhanced sensitivity to RE, however, has not been identified in another two AT-like radiosensitive mutant lines, V-C4 and V-G8, which are in the same complementation group as *irs*-2 (Natarajan et al 1992). The yield of chromosomal aberrations after *Alu* I and *Bam*H I treatment were found to be similar in both V-C4 or V-G8 cell lines to those obtained in the wild-type parental V79 cell lines (Natarajan et al 1992). Therefore, whether dsb are the primary lesions responsible for the cytogenetic anormalies following ionizing radiation observed in these mutant cell lines, which show a normal ability to rejoin dsb, is presently unclear.

While preparing this manuscript, Costa and Thacker (1993) reported the survival response to RE of several human radiosensitive cell lines, including AT5BIVA, 46BR1G1 (a ligase I deficient cell line) and GM8505 (a BS cell line). They found that AT cells exhibited a highest sensitivity to the killing effect of *Pvu* II, followed by 46BR1G1 cells, while GM8505 cells were no more sensitive to *Pvu* II than normal fibroblast cells (MRC5V1). All the radiosensitive cell lines examined showed a normal cellular sensitivity to *Ban* I (induces cohesive-ended dsb) when compared to the normal cell line (Costa and Thacker 1993). The data suggest that the sensitivity of the cell lines to ionizing radiation correlates to their sensitivity to blunt-ended dsb, but not to cohesive-ended dsb (Costa and Thacker 1993).

1. 3. Purpose of Present Study

As previously discussed, AT cells are hypersensitive to ionizing radiation and some DNA damaging agents, but the nature of the primary

lesions to which AT cells are sensitive remains unclear. Although evidence strongly suggests that DNA strand breakages are the lesions responsible, this has not been supported by biochemical studies which determined a normal gross rejoining of ssb and dsb in AT cells. The defect in AT has been proposed to relate to a low fidelity of DNA repair (Cox 1982) which may also relate to an increased chromosomal instability in AT cells. It is important to know what is the primary damage that leads to hypersensitivity and other abnormal responses of AT to ionizing radiation and how this damage is specifically processed in AT cells.

On the basis of understanding that dsb play a major role in cell lethality and chromosomal aberrations and that RE-induced dsb mimic radiation effects on cells, the present study has focused on the clastogenic sensitivity and other responses of AT cells to dsb generated in DNA by RE. The main aim of the study was to investigate the possible hypersensitivity of AT cells to a specified lesion, namely the dsb, and to establish a relationship between chromosomal instability and the putative hypersensitivity to dsb in AT cells thereby providing evidence for the existence of an underlying defect in AT cells in the processing of dsb.

The strategy of this study was to investigate 5 specific aspects of the response of AT cells. Firstly, to characterise the sensitivity of the AT and human normal lymphoblastoid cell lines to ionizing irradiation. This has not been done before with these cell lines and the great heterogeneity generally found among AT cell lines necessitated this study. The radiation responses of AT and normal cells studied here include an investigation of the induction and rejoining of dsb, the induction of chromosomal aberrations, the kinetics of chromosomal aberrations generated in G_2 cells (G_2 assay), the response of DNA synthesis, and cell cycle disturbance in AT and normal cell lines. This part of study is presented in Chapter 3.

Secondly, a comparative investigation of the chromosomal sensitivity of AT and normal cells to RE-induced dsb has been carried out; described in Chapter 4. RE which cause blunt- or cohesive-ended dsb were used in order to test the clastogenicity of dsb with different end-structures. In addition, considering that DNA cleavage is a critical factor, some factors relevant to the action of RE inside cells, such as cell poration efficiency and the activity and stability of RE under simulated cellular conditions were examined.

The third aspect of this study was designed to examine the potentiation of a powerful DNA repair inhibitor, ara A, on the chromosomal aberrations originating from RE-induced dsb with either blunt- or cohesive-end structures. These experiments attempted to investigate the repair of dsb generated by RE which may relate to the conversion of unrepaired or misrepaired dsb into chromosomal aberrations. This study is presented in Chapter 5.

Fourthly, since the resistance of DNA synthesis to irradiation is one of the characteristic hallmarks of AT, DNA replication after RE treatment has been investigated in normal and AT cells. This approach was an attempt to test the hypothesis that dsb is a primary lesion responsible for the induction of inhibition of DNA synthesis. The influence of the end-structure of dsb on the induction of normal inhibition of DNA synthesis were examined and these results are presented in Chapter 6.

Finally, the possibility of introducing normal protein extracts into AT cells to restore the chromosomal sensitivity of AT cells to γ-irradiation and RE, has been tested, in an attempt to search for a possible protein factor(s) which may be involved in the defect in AT cells. This is discussed in Chapter 7.

Chapter II

Materials and Methods

2. 1. Cell culture

2. 2. Purification of restriction endonucleases

2. 3. Measurements of activity and stability of restriction endonucleases

2. 4. Cell poration with streptolysin-O (SLO)

2. 5. Poration efficiency assay

2. 6. Irradiation with X- or γ-rays

2. 7. Micronucleus assay

2. 8. Chromosome preparation and scoring of metaphase aberrations

2. 9. Determination of DNA double strand breaks

2. 10. DNA synthesis assay

2. 11. Flow cytometric analysis of cell cycle

2. 12. Preparation of cell extracts

2. 1. Cell culture

Human lymphoblastoid cell lines derived from two AT female homozygote individuals, AT-PA and AT-KM, and from a normal female donor, N-SW, have been used in the study. They were immortalized by Epstein-Barr virus transformation and were kindly supplied by Dr. A.M.R. Taylor (University of Birmingham, UK).

Cells were maintained as suspension cultures in RPMI 1640 medium at 37 °C in an atmosphere of 5% CO_2. The medium was supplemented with 2 mmol/l L-glutamine (Gibco-BRL), 8.3% (v/v) foetal calf serum (Globepharm), 8.3% (v/v) tryptose phosphate broth (Gibco-BRL), 50 units/ml penicillin and 50 µg/ml streptomycin (Sigma). Cells were routinely passaged every three days, by seeding cells at approximately 3×10^5 cells/ml. Cell numbers were determined using an electronic particle counter (Coulter Counter), alternatively maintenance of cell cultures was simply done by diluting by a factor of 3 with medium. For experimentation cells were passaged 1 - 2 days prior to use at 3 to 5×10^5 cells/ml and the cells used during exponential growth. All experiments were performed with asynchronous cell populations.

All the cell lines are of near-diploid karyotype and the chromosome numbers per cell were mainly found to range from 43 to 46 for AT-PA, AT-KM and N-SW cell lines. As shown in Figure 2. 1, over 80% of cells have 44 to 46 chromosomes. The doubling-times for AT-PA and N-SW cell lines are similar and estimated to be approximately 24 hours as measured the increase of cell number over several days (Figure 2. 2), while the measurement of cell number doubling time for the AT-KM cell line was not valid since a lot of cells die while cell growing.

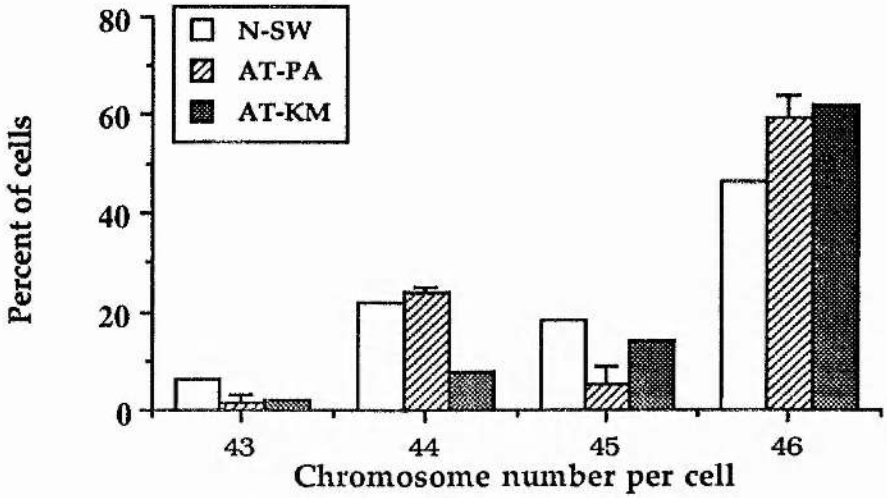

Figure 2.1. Ranges of chromosome numbers of AT-PA, AT-KM and N-SW cell lines. Data for AT-KM and N-SW cells were obtained by scoring 50 metaphases. Data for AT-PA cells were derived from 200 metaphases in 2 independent chromosome preparations (average values ± standard errors).

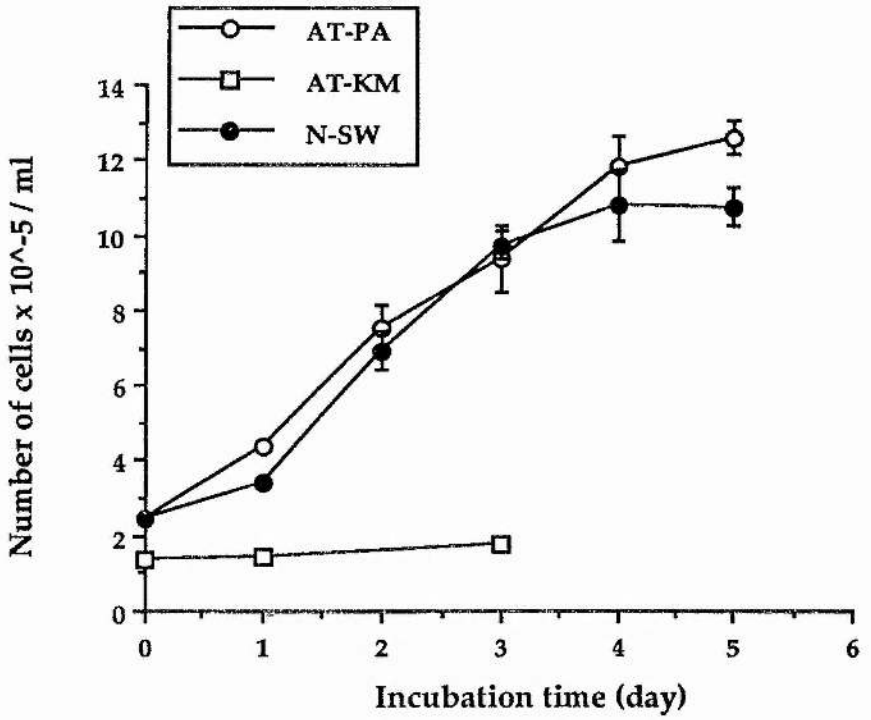

Figure 2. 2. Cell growth curves in RPMI 1640 medium at 37 °C for AT-PA, AT-KM and N-SW cells. Average values and standard deviations were obtained from 2 determinations.

2. 2. Purification of restriction endonucleases

2. 2. 1.Restriction endonucleases

All the restriction endonucleases (RE) used in the studies, *Pvu* II, *Bam*H I, *Pst* I, *Eco*R I and *Eco*R V (all purchased from Gibco-BRL or NBL) recognize and cleave sequences of six bases in DNA and cause either blunt or 4-base overhang in either 3′- or 5′-direction Their characteristics are shown in Table 2. 1.

Table 2. 1. Characteristics of the restriction endonucleases used

Enzyme	Recognition sequence[a]	End-structure generated	Average base pairs between cutting sites[b]
Pvu II	CAG/CTG	Blunt	3212
*Eco*R V	GAT/ATC	Blunt	5123
Pst I	CTGCA/G	3'-overhang	3212
*Bam*H I	G/GATCC	5'-overhang	5533
*Eco*R I	G/AATTC	5'-overhang	3013

[a] The symbol "/" indicates the cleavage site of RE in the 5′--3′ strand of the DNA double helix.

[b] Calculated according to Bishop et al (1983).

2. 2. 2. Purification of restriction endonucleases

Enzymes were purified free of storage buffer by ultrafiltration using Amicon 10 filters (Amicon) in the presence of Hank's balanced salt solution (HBSS), as described previously (Bryant and Christie 1989). All the procedures for purification were carried out on ice or at 4 °C. The filters were sterilized with 70% (v/v) ethanol and washed with sterilized distilled water. The required amount of enzyme was added to the filter after addition of 50 µl of HBSS containing 1% (w/v) bovine serum albumin (BSA, BDH), and diluted with 1 ml of HBSS. The mixed solution was centrifuged at 8,000 rpm (7,700 x g) in a JA 20 rotor (Beckman) for 60 min followed by a further centrifugation for 90 min after the addition of another 1 ml of HBSS. The residual solution above the filter was collected by centrifugation at 2000 rpm for 5 min and the enzyme concentration was adjusted to 1 unit/µl (or in some experiments to 10 units/µl) using HBSS containing 1% BSA (HBSS/BSA). Purified enzymes were used immediately or in some instances stored at 4 °C overnight before use.

2. 3. Measurements of activity and stability of restriction endonucleases

2. 3. 1. RE activity assay

The activity of RE before and after ultrafiltration was measured with a titration assay using plasmid pBR322. Evaluation of the activity assay was made by titration of RE before purification following instructions of manufacturer's (USB). *Bam*H I and *Pvu* II (0.06 to 1 unit) were mixed with 1 µg of pBR322 (Pharmacia) in a total assay buffer volume of 50 µl and

incubated at 37 °C for 1 hour. The minimum amount of enzyme required for complete digestion of a circular form of the plasmid to a linearized form was determined. Figure 2. 3 shows that both *Bam*H I and *Pvu* II at 1 unit completely linearized the plasmid. This is consistent with the manufacturer's unit definition of the enzymes.

The ability of the RE to digest pBR322 after purification was investigated either in their optimum reaction buffers (Gibco-BRL) or in cell extracts which were used to simulate cellular ionic conditions. The extracts were prepared as described in 2. 12. 1 and boiled before use to eliminate the interference of enzymes in the extracts with the plasmid. 2 µl of the enzyme dilution in HBSS/BSA were added to a reaction mixture of 12 µl, containing 0.35 µg of pBR322 (Gibco-BRL) and either the cell extracts or buffer. After incubation at 37 °C for 1 hour, the reactions were terminated by the addition of SDS (Sigma) at 1% (w/v) and stored at -20 °C. The samples were electrophoresed on a 0.8% (w/v) agarose (Sigma) gel containing 0.5 µg/ml of ethidium bromide (EB, Sigma) in a Tris-borate-EDTA buffer (TBE, containing 89 mmol/l Tris-borate, pH 8.3, and 2.5 mmol/l EDTA, purchased from NBL), at a constant voltage overnight. The amount of RE required for the complete digestion of the plasmid was determined.

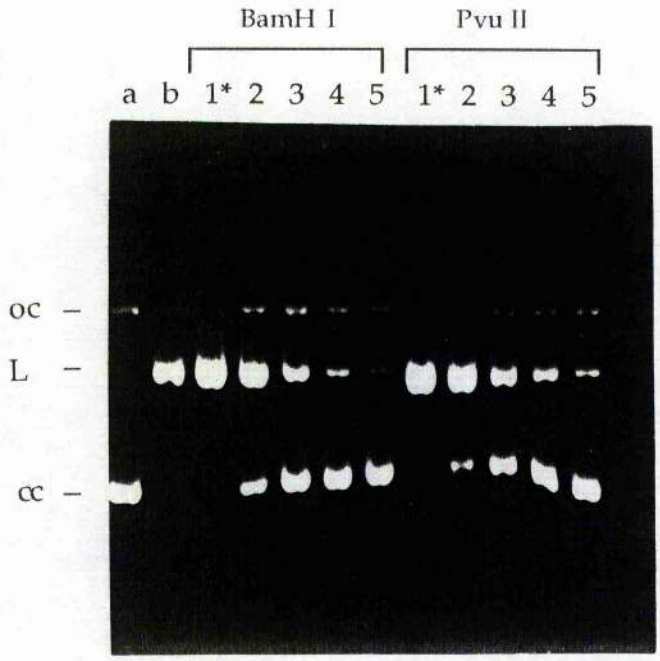

Figure 2.3. Activity assay for *Bam*H I and *Pvu* II before purification. Lane a: pBR322; lane b: linearized pBR322. Lanes 1 to 5: enzyme 1, 0.5, 0.25, 0.125 and 0.06 units, respectively. cc: closed circles; L: linear; oc: open circles. * RE amount for complete digestion of pBR322.

The activity of RE in the reaction buffer and that in the cell extracts were compared and the results for *Bam*H I is shown in Figure 2. 4. The amount of *Bam*H I required for complete digestion of the plasmid is 0.25 units for the reaction carried out in the cell extracts and 1 unit for that in the buffer (Figure 2. 4). The activity of the enzyme in the cell extracts thus appeared to be higher than that in the buffer. In addition, as shown in Figure 2. 5, it was observed that the presence of BSA during ultrafiltration and plasmid digestion was necessary for acquiring and maintaining a higher activity of RE.

2. 3. 2. RE stability assay

The stability of the enzymes after purification was examined by observing the maintenance of their activities after storage at either 4 °C in HBSS/BSA or 37 °C in the cell extracts. RE (5 to 10 µl in HBSS/BSA at 1 unit/µl) were mixed with an equal volume of the boiled cell extracts and incubated at 37 °C for various times. Following incubation, the enzymes were diluted with HBSS/BSA and the activities measured as described in 2.3.1.

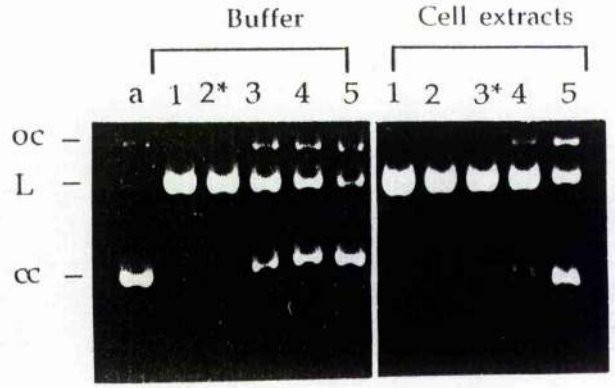

Figure 2.4. Comparison of *Bam*H I activity assayed in reaction buffer and in boiled cell extracts. Lane a: pBR322. Lanes 1 to 5: enzyme 2, 1, 0.5, 0.25 and 0.125 units, respectively. cc: closed circles; L: linear; oc: open circles. * RE amount for complete digestion of pBR322.

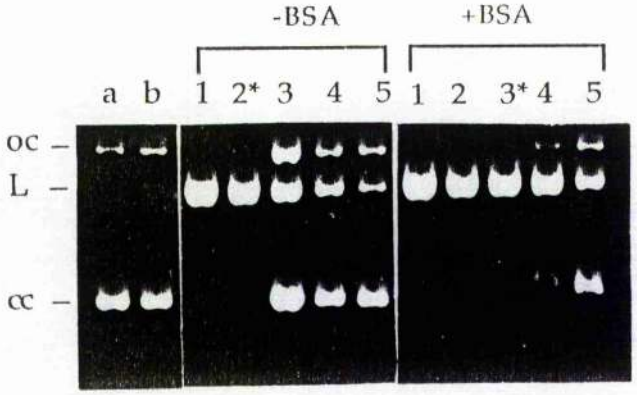

Figure 2.5. Comparison of the activity of *Bam*H I purified in the absence and in the presence of BSA. The assay was carried out in the cell extracts at 37 °C for 1 hour. Lane a: pBR322; lane b: pBR322 incubated with the the extracts for 1 hour. Lanes 1 to 5: enzyme 2, 1, 0.5, 0.25 and 0.125 units, respectively. cc: closed circles; L: linear; oc: open circles. * RE amount for complete digestion of pBR322.

2. 4. Cell Poration with streptolysin-O (SLO)

The introduction of RE into cells was achieved by porating cells with the reduced bacterial toxin streptolysin-O (Wellcome Diagnostics). Cell poration was carried out essentially as previously described for Chinese hamster cells (Bryant 1992). SLO was dissolved as *per* the manufacturers' recommendations, usually giving a stock solution of 1.9 units/ml, which was stored in aliquots (0.5 ml) at -20 °C. One unit of SLO is defined by the manufacturers as the amount of the toxin causing 50% lysis of 2% erythrocyte suspension in phosphate buffered saline (PBS) pH 7.4 after incubation at 37 °C for 30 min. Cells grown in suspension were centrifuged at 1,200 rpm for 5 min at 4 °C and cell pellets resuspended in HBSS at 1 to 3 x 10^6 per ml depending on the requirement of a particular experiment. Cells were mixed with the required amount of SLO and RE in a 10 ml conical-bottomed centrifuge tube at a final concentration of 1 x 10^6 cells/ml. The exposure of cells to SLO and RE was for 5 min at room temperature (about 20 °C) and was terminated by the addition of 5 ml of RPMI 1640 medium and centrifugation at 1,200 rpm for 5 min. The cell pellets were then resuspended in fresh RPMI 1640 medium.

2. 5. Poration efficiency assay

To examine the effects of SLO on cell membrane poration, the release of ^{3}H-methionine labelled cellular proteins through the cell membrane was determined. Cells were seeded at 5 x 10^5 cells/ml in RPMI 1640 medium and incubated with ^{3}H-methionine (Amersham International) at 3.7 x 10^4 Bq/ml for 24 hours. The labelled cells were centrifuged at 1,200 rpm for 5 min,

washed once with the medium and chased in non-radioactive RPMI 1640 medium at 37 °C for 24 hours. The cells were then harvested in HBSS and treated with SLO at various concentrations for 5 min or at 0.06 units/ml of SLO for various times. Cells were pelleted by centrifugation and whole supernatants (0.5 ml each) were loaded on Whatman No. 3 filter discs , dried at 60 °C and radioactivity counted by liquid scintillation. In some experiments, the supernatants were ultrafiltered with Amicon 10 filters (which retain molecules with MW greater than 10 Kdal) and ^{3}H-activities in the filtrate and on the filter were determined.

2. 6. Irradiation with X- or γ-rays

Cells suspended in aerated medium were irradiated with x- and γ-rays at dose rates of approximately 1 Gy/min and 4.6 Gy/min, respectively. X-rays were generated by a Marconi therapy set operating at 250 KV, filtered with a 0.5 mm copper filter. γ-irradiation was carried out in a ^{137}Cs IBL437C γ-irradiator (CIS UK Bio International). Irradiations were performed at room temperature for cytogenetic studies or on ice for measurement of DNA double strand breaks (dsb). Dosimetry was checked by a ferrous sulphate method (Frankenberg 1969).

2. 7. Micronucleus assay

Micronuclei (Mn) were assayed by the cytokinesis block technique by using Cytochalasin B (Fennech and Morely 1985). Cytochalasin B (CYT-B, Sigma) was made up as a stock solution at 3 mg/ml in dimethyl sulphoxide (Sigma) and added to cell cultures at 3 μg/ml immediately following X-

irradiation or RE treatment to block the cytokinesis of cells. The cells which reach the first mitosis would consequently appear as binucleated (BN) cells. Mn were scored only in BN cells. For the Mn assay cells were incubated at 37 °C for various times and approximately 10^5 cells subsequently spread on slides by spinning the cell suspension in a Cytospin 2 centrifuge (Shandon) at 800 rpm for 10 min. The slides were air dried, fixed with methanol for 10 min, dried and stained in 10% Giemsa (BDH) in tap H_2O for 45 min. The frequencies of micronuclei were scored in 100 BN cells for each sample at a magnification of 10 x 100 under a light microscope (Zeiss).

The effect of CYT-B on cytokinesis was examined and the results are illustrated in Figure 2. 6. Incubation of cell with various concentrations of CYT-B resulted in an increased frequencies of BN cells in AT-PA as well as in N-SW cell lines and similar levels of BN% obtained at 3 μg/ml of CYT-B in both cell lines (Figure 2. 6). The SLO (0.06 units/ml) treated cells exhibited lower frequencies of BN cells when compared with the untreated cells (Figure 2. 6). The stability of CYT-B at 37 °C was also examined by incubation of CYT-B at 3 μg/ml in RPMI 1640 medium for various period of time prior to application to cell culture, and subsequently determination of BN% of cell culture following 48 hours incubation. Figure 2. 7 shows that the effects of CYT-B are maintained at the same level (producing approximately 50% BN cells) after 1 to 6 days of previous incubation in medium.

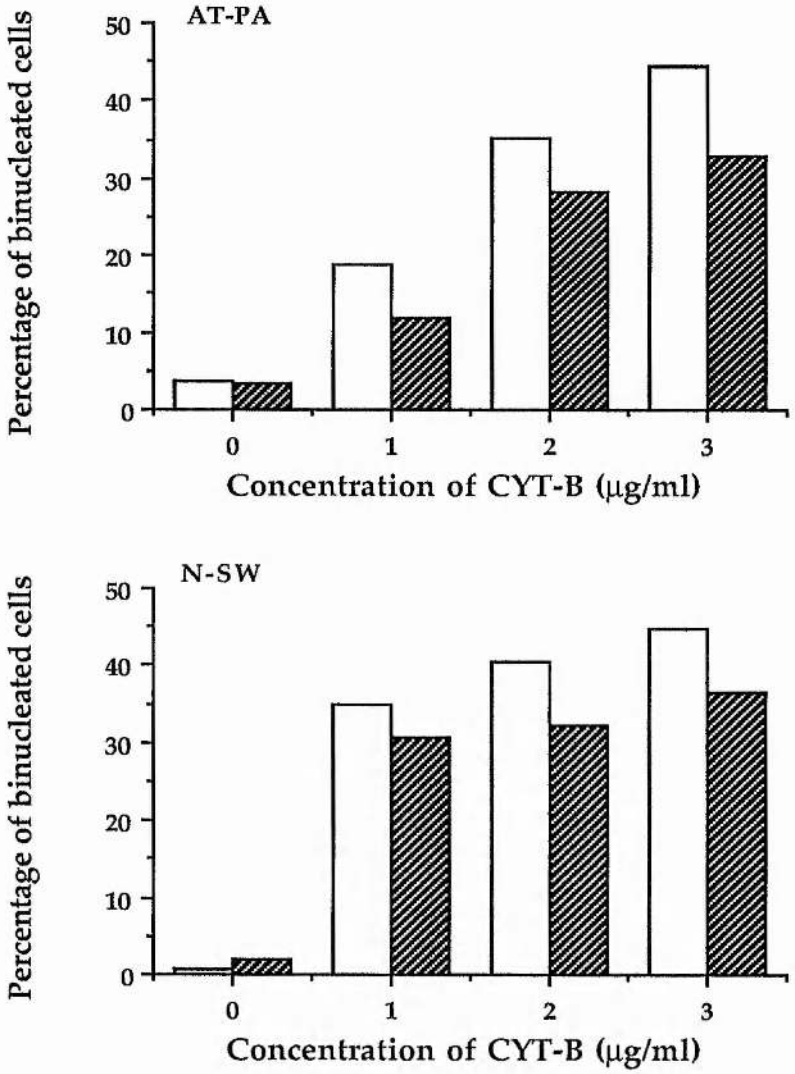

Figure 2.6. Frequencies of binucleated cells as a function of CYT-B concentrations in SLO treated (striped bars) or untreated (blank bars) cells of AT-PA (upper panel) and N-SW (lower panel) lines after incubation at 37 °C for 48 hours.

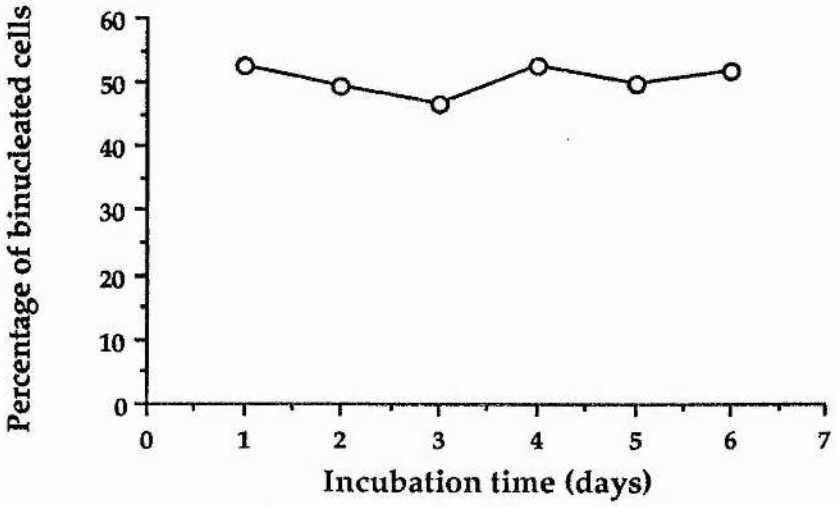

Figure 2.7. Percentage of binucleated cells in N-SW cells after a 48-hour incubation with CYT-B (3 μg/ml) which was previously incubated in the medium at 37 °C for 1 to 6 days.

2. 8. Chromosome preparation and scoring of metaphase aberrations

2. 8. 1.Chromosome preparation

To obtain enough metaphase cells for chromosomal study, colcemid (stock solution: 4μg/ml; Sigma) was added to cell culture 3 to 4 hours before harvesting at 0.04 μg/ml to accumulate cells at metaphase. Cells were collected by centrifugation at 1,200 rpm for 5 min. The supernatant was aspirated and the cells were resuspended in 0.075 mol/l KCl at room temperature for a hypotonic treatment for 3 min. After centrifugation and removal of the supernatant, the cell pellets were flicked to resuspend cells which were then fixed in fresh Carnoy's fixative (methanol: acetic acid 3:1). Fixation of cells was repeated at least three times. The cells could be stored in the fixative at 4 °C for several months. For metaphase spreads, cells were

resuspended in a few drops of fresh fixative, dropped on to -20 °C chilled slides and warmed by hand immediately.

Glass slides (BDH) used for chromosome spreading were pre-cleaned to obtain better chromosome preparations. The slides were treated with HCl solution (approximately 1 mol/l) overnight and washed with distilled H_2O and then absolute ethanol for 2 hours. The slides were dried and chilled in a -20 °C freezer prior to use. In some experiments, the chilled slides were wetted with few drops of 50% (v/v) acetic acid immediately before the cells were applied. This was found to give better metaphase spreads and to prevent "fuzzy" chromosomes. The slides were air dried and stained with 3% (v/v) Giemsa in tap H_2O for 20 min.

2. 8. 2. Analysis of metaphases for aberrations

Scoring for chromosome aberrations was carried out under a Zeiss microscope with a oil immersion objective (magnification of 12.5 x 63). The aberrations were defined as chromatid- and chromosome-type based on the classifications of Buckton and Evans (1981).

Chromatid-type aberrations included chromatid gaps, chromatid breaks and chromatid exchanges. Chromatid gaps and breaks are both discontinuous regions in a chromatid. Any region which was not greater than the diameter of the chromatid was scored as a gap, while that which was greater than the diameter was scored as a break. Chromatid exchanges included asymmetrical or symmetrical quadriradials and triradials.

Chromosome-type aberrations included chromosome exchanges (dicentric or polycentric chromosomes and rings), chromosome deletions and chromosome gaps. A polycentric (n centric) chromosome was scored as

an n-1 chromosome-type exchange. An acentric fragment which was not accompanied by a dicentric chromosome or a centric ring was scored as a chromosome deletion.

2. 9. Determination of DNA double strand breaks

Double strand breaks (dsb) were measured by "neutral" filter elution at pH 9.6 as described by Bradley and Kohn (1979) and modified by Okayasu and Iliakis (1989). Cells at 5 x 10^5/ml in medium were pre-labelled with ^{3}H-thymidine (Amersham International) at 3.7 x 10^4 Bq/ml overnight. After irradiation or treatment with RE, the cells were immediately diluted in 20 ml of pre-cooled (4 °C) phosphate buffered saline (PBS) in a 40 ml syringe barrel connected to a Millipore (Swinnex) containing a Nucleopore filter (pore diameter: 2μm) by gravity flow. The cells on the filter were then lysed at 60 °C for 60 min to release DNA by the exposure of cells to 1 ml of lysing buffer (pH 9.6): 0.025 mol/l sodium EDTA (BDH), 0.1 mol/l glycine (BDH) and 0.068 mol/l N lauryl sarcosine (Sigma). DNA in the lysis solution was eluted at room temperature with 40 ml of a buffer containing 0.02 mol/l Tris and 0.05 mol/l sodium EDTA, pH 9.6, over a period of approximately 16 hours at a controlled flow rate regulated by a peristaltic pump (Watson Martor). The eluants were collected in 25 cm^2 flasks and weighed to determine total volume eluted. 5 ml of the eluant was mixed with 6 ml Optiphase^{TM} (LKB) and the radioactivity determined in a liquid scintillation counter (LKB Wallac). Filters were counted in 4 ml of Filter-Counter^{TM} (Packard). The total radioactivity eluted was calculated by formula (1).

$$T_{(eluted)} = [\ dpm_{(elution)} / 5\ ] \times V \qquad (1)$$

where $T_{(eluted)}$ = total radioactivity eluted; $dpm_{(elution)}$ = dpm measured for 5 ml elution; V = total volume eluted.

The fraction of DNA eluted was then calculated as a ratio of total radioactivity eluted to total radioactivity on the filter and in the eluant. The fraction of DNA eluted was calculated as formula (2):

$$F = T_{(eluted)} / [T_{(eluted)} + dpm_{(filter)}] \qquad (2)$$

where F = fraction of DNA eluted; $T_{(eluted)}$ = total radioactivity in the elution; $dpm_{(filter)}$ = dpm measured on the filter.

The ratio of dsb rejoined was calculated using formula (3):

$$\text{Ratio of dsb rejoined} = 1 - (F_i - F_c) / (F_o - F_c) \qquad (3)$$

where F_i is the fraction of DNA eluted for irradiated cells after incubation; F_c is the fraction of DNA eluted for the unirradiated cells; F_o is the fraction of DNA eluted immediately after irradiation.

2. 10. DNA synthesis assay

The rate of incorporation of ^{3}H-TdR into DNA by cells was determined for assay of DNA replication. Cells were suspended in medium (0.5 to 1 x 10^6/ml) and ^{3}H-TdR was added at a final concentration of 3.7 x 10^4 Bq/ml (1 μCi/ml). The incubation was carried out in a water bath at 37 °C for

30 min or 60 min. Reactions were terminated by the addition of 5 ml of ice cold HBSS/BSA and placing tubes on ice. Cells were centrifuged at 4 °C and cell pellets resuspended and lysed with 1 ml of 0.03 mol/l NaOH at room temperature for 10 min. DNA was precipitated with trichloroacetic acid (TCA; purchased from BDH) at a final concentration of 0.31 mol/l at 4 °C overnight. The acid-precipitated DNA was collected on Whatman GF/C fibreglass filters, washed with 0.31 mol/l TCA and absolute ethanol. The filters were dried at 60 °C and dpm counted in 4 ml Filter-Count™ in a liquid scintillation counter. The incorporation of ^{3}H-TdR was normalized as a dpm per 10^5 cells and the rate of DNA synthesis was determined as a ratio of ^{3}H-TdR incorporation in the treated cells to that in untreated control cells.

2. 11. Flow cytometric analysis of the cell cycle

Cells were harvested by centrifugation at 1200 rpm for 5 min and the supernatant aspirated. Cell pellets were resuspended and fixed in 70% (v/v) ethanol for 30 min or overnight at 4 °C. Following a single wash with PBS, cells were resuspended at 1 x 10^6/ml in a fresh solution of 1 mg/ml RNase A (Sigma) in PBS and incubated for 30 min at 37 °C before the addition of propidium iodide (Sigma) at 4 μg/ml. Single cell suspension were obtained by resuspending cells and passing them through a No. 21 gauge needle. Data for the measurements by forward scatter versus fluorescence for propidium iodide were acquired in a flow cytometer (FACScan, Becton Dickinson) using a software "Consort #30", and the populations of the cells in G_1, S and G_2 phases were analysed using "DNA" software.

2. 12. Preparation of cell extracts

2. 12. 1. Preparation of whole cell extracts

Whole cell extracts were prepared as described by Mohamed and Lavin (1986) with some modifications. All the following procedures were performed on ice or at 4 °C.

(a). Cell homogenisation

Cells growing exponentially were harvested by centrifugation at 1200 rpm for 5 min and washed twice with cold extraction buffer:

Hepes buffer (Gibco-BRL) 40 mmol/l, pH 7.0
KCl 80 mmol/l
Dithiothreitol (DTT, Sigma) 1 mmol/l
EGTA (Sigma) 1 mmol/l
$MgCl_2$ 4 mmol/l
ATP (Sigma) 2 mmol/l

Cells were resuspended at approximately 2 x 10^7 cells/ml in the extraction buffer supplemented with phenylmethylsulphonyl fluoride (PMSF), an inhibitor of serine protease, at 0.1 mmol/l, homogenized in a Dounce homogenizer with an "A" pestle for 45 strokes and the homogenates checked under microscope. A volume of KCl (stock solution 5 mol/l) was added to the homogenates to adjust the concentration of KCl to 380 mmol/l. The homogenates were then held for 30 min with periodic gentle agitation followed by ultracentrifugation at 23,000 rpm (54,000 x g) in a 70Ti rotor (Beckman) for 60 min.

(b). Precipitation of extract protein

The supernatants were collected and solid $(NH_4)_2SO_4$ (BDH) added to give 70% saturation. After 30 min, the proteins were precipitated and pelleted by centrifugation for 30 min at 15,000 rpm (27,000 x g) in a JA20 rotor (Beckman). The protein pellets were redissolved in 1 ml of the extraction buffer per 2.7×10^7 cells originally used.

c). Dialysis of the extracts

The extracts were extensively dialysed against the extraction buffer containing 300 mmol/l sucrose (BDH) at 4 °C overnight with at least 3 changes of the buffer with vigorous agitation using a magnetic stirrer. To remove the precipitated proteins after dialysis, the extracts were collected in 1.5 ml Eppendorf tubes and centrifuged at 12,000 rpm (18,600 x g) in a JA 18.1 rotor (Beckman) for 20 min. The supernatants were collected in Eppendorf tubes and stored at -20 °C.

2. 12. 2. Preparation of nuclear extracts

Extraction of nuclear proteins from human lymphoblastoid cells was performed as described previously by North et al (1990) with some modifications. All the following procedures were carried out on ice or at 4 °C. The buffers used are listed as following:

Buffer A: Tris-HCl 20 mmol/l, pH 7.5
$MgCl_2$ 0.5 mmol/l
$CaCl_2$ 2 mmol/l
KCl 0.5 mmol/l

Buffer B: NaCl 500 mmol/l
EDTA (sodium salt) 10 mmol/l

Buffer C: Tris-HCl 50 mmol/l, pH 7.5
EDTA (sodium salt) 0.1 mmol/l
2-Mercaptoethenol 10 mmol/l

(a). Isolation of nuclei

Exponentially growing cells were used for preparation of nuclear extracts. In specific experiments cells, suspended in RPMI 1640 medium containing 10% (v/v) of DMSO, were frozen in liquid nitrogen before use. The cells were rapidly thawed at 42 °C and pelleted at 1,200 rpm for 5 min. The cell pellets were washed twice with HBSS/BSA, twice with HBSS and once with buffer A1 (buffer A supplemented with 0.85 g of sucrose in 10 ml). The cells were held in buffer A1 for 15 min and resuspended in buffer A2 (buffer A supplemented with 0.1 mmol/l PMSF) following centrifugation. The cells were homogenized in a Dounce homogenizer with a "B" pestle for 40 strokes. The nuclei were pelleted and washed with buffer A2 for 4 times by centrifugation at 2,000 rpm.

(b). Extraction of nuclear proteins

The nuclei were resuspended at 1 x 10^7 nuclei/ml in buffer B supplemented with 0.1 mmol/l of PMSF and held on ice for 30 min with gentle agitation. Following centrifugation at 2,500 rpm for 5 min the supernatants were collected in Eppendorf tubes and centrifuged at 12,000 rpm (18.600 x g) in JA18.1 rotor for 30 min.

(c). Desalination of the extracts

The supernatants were desalted by ultrafiltration at 8,000 rpm (7,700 x g) through Amicon 10 filters and washed once with 1 ml of buffer C by the

same procedure. The residual solution containing nuclear proteins was collected by centrifugation at 2,000 rpm and dispensed in to Eppendorf tubes and stored at -20 °C. Once thawed, re-freezing of the extracts was avoided.

2. 12. 3. Protein assay

The levels of protein in extracts were determined by the Bio-Rad microassay procedure as *per* the manufacturers' instruction. For each assay, a protein standard curve was produced by diluting a standard solution of BSA (stock solution: 100 µg/ml) in distilled water to 0.8 ml and adding 0.2 ml of Dye Reagent Concentrate (Bio-Rad). The extracts (2-10 µl) or the same volume of buffer (reagent blank) were diluted with distilled water to 0.8 ml in 1.5 ml Eppendorf tubes to which 0.2 ml of Dye Reagent Concentrate were added. After vortex mixing the OD values were measured versus reagent blank in a spectrophotometer at a wavelength of 595 nm. The unknown protein concentrations of extracts were read from the standard curve so produced.

Chapter III

Characterization of Radiosensitivity of AT-PA, AT-KM and N-SW Cell Lines

3. 1. Introduction

3. 2. Results

3. 2. 1. Chromosomal sensitivity to ionizing irradiation as measured by micronuclei production

3. 2. 2. Chromosomal aberrations induced by γ-irradiation

3. 2. 3. Kinetics of chromosomal aberrations in G_2 phase cells

3. 2. 4. Chromosomal sensitivity to bleomycin

3. 2. 5. Induction and rejoining of double strand breaks in DNA

3. 2. 6. Cell cycle response

3. 2. 7. DNA synthesis

3. 3. Discussion

3. 1. Introduction

Individuals with the autosomal recessive disease ataxia telangiectasia are hypersensitive to ionizing radiation and radiomimetic DNA damaging agents. The characteristic features of AT cells in response to ionizing radiation include an enhanced proliferative cell death, an increased induction of chromosomal aberrations, a reduced inhibition of DNA synthesis and an abnormal cell cycle disturbance (reviewed in Chapter 1). These abnormal responses of AT cells to ionizing radiation seem to be the result of a dysfunction of the AT gene which has been localised to chromosome 11q22-23 (Gatti et al 1989). The association between the specific genetic alterations and the increased killing effect of ionizing radiation on AT cells is unknown. A general acceptance of this association is that a defect in the repair of DNA damage is involved in AT; however the precise biochemical basis of the defect is not clear. AT cells are consistently more sensitive to agents such as X-irradiation and bleomycin which directly produce strand-breaks in the DNA of cells, suggesting that AT cells are defective in the processing of DNA breaks (Shiloh et al 1985). However, the production and rejoining of ssb and dsb induced by ionizing radiation and radiomimetic agents was not found to be different in most AT cell lines from those in normal cells, as determined by any of the assays developed so far.

AT cells exhibit an enhanced frequency of radiation-induced chromosomal aberrations in the G_0 (G_1) and the G_2 phases of the cell cycle and there is an unusual occurrence of chromatid-type aberrations in cells exposed to ionizing radiation in the G_0 (G_1) phase (Taylor 1976, 1978, Natarajan and Meyer 1979). Although it is generally accepted that chromosomal aberrations arise from dsb in DNA, the precise mechanisms of chromosomal aberrations are not understood, neither is the underlying

basis of the increased chromosomal sensitivity of AT cells. Investigation of the radiosensitivity of AT cells may prove to be a useful strategy with which to identify the processes and sequence of events that lead from the initial induction of DNA damage to chromosomal aberrations and ultimately to proliferative cell death.

It has been suggested that although bulk dsb are rejoined normally in AT cells, a small fraction of unrepaired dsb, which may be undetectable by current techniques, as well as misrepaired dsb, are likely to be responsible for chromosomal aberrations (Taylor 1978). Accumulating evidence has shown that AT cells possess a higher probability of dsb misrepair when compared with normal cells (Cox et al 1984, Debenham et al 1988, North et al 1990, Miyajima et al 1993, Powell et al 1993). Studies of the kinetics of chromosomal breaks, as measured by the disappearance of PCC fragments in G_0 cells, showed an enhanced level of residual chromosomal damage after proficient initial repair of damage (Cornforth and Bedford 1987). On the other hand, the kinetics of disappearance of chromatid aberrations induced by irradiation in G_2 cells has shown that normal chromosomal repair occurs in AT cells while the conversion of DNA damage into chromosomal aberrations is greater in AT cells than in normal cells (Mozdarani and Bryant 1989a). Because the dose-range used in the determination of chromosomal damage is similar to that used for cell survival and much less than that used for biochemical assays, these results are more likely to accurately represent underlying DNA repair mechanisms.

In most cases, the reduction in the inhibition of DNA synthesis and the altered progression through the cell cycle of irradiated AT cells appear to be associated with their increased radiosensitivity, while there is evidence that in individual AT cell lines the reduced inhibition of DNA synthesis is not always associated with an enhanced cell killing and chromosomal aberrations (Taylor et al 1987). The relationship between cell lethality,

chromosomal instability, DNA replication and cell cycle alterations remains to be established.

AT cells have been shown to be very heterogeneous, both for the clinical manifestations and for the cellular response to ionizing radiation. Different AT cell lines may differ in their sensitivity to ionizing radiation or to bleomycin (Hittleman and Sen 1988). In fact, altered repair of dsb induced by ionizing radiation and bleomycin has been found in one AT cell line (Coquerelle and Weibzahn 1981, Coquerelle et al 1987). Furthermore, transformed or immortalised lymphoblastoid AT cell lines have been shown to differ in many respects from primary lymphocytes or fibroblasts, such as spontaneous chromosomal instability (Cohen and Simpton 1980), radiation-induced chromosomal aberrations, initial levels of PCC breaks (Coquerelle et al 1987, Pandita and Hittleman 1992) and the response of DNA supercoiling to ionizing radiation (Taylor et al 1991).

The work presented in this chapter examines whether the AT cell lines used in this study display the features of chromosomal hypersensitivity and of the responses of cell cycle and DNA replication to ionizing radiation. The relationship between dsb and chromosomal aberrations induced by radiation has already been discussed.

3. 2. Results

3. 2. 1. Chromosomal sensitivity to ionizing irradiation as measured by micronuclei production

The chromosomal sensitivity of AT and normal cells to ionizing radiation was investigated by the micronuclei (Mn) assay using the cytokinesis block technique. Figure 3. 1. shows the results of Mn induction in AT-PA and N-SW following various doses of X-ray and sampled at

various incubation times after irradiation. Following 1 and 2 Gy of X-ray, the frequencies of Mn in AT-PA cells increased with incubation time to reach a maximum yield of Mn at 72 h (Figure 3. 1). N-SW cells showed lower levels of Mn induction for each dose of radiation and after each incubation time when compared with AT-PA cells, indicating an enhanced chromosomal sensitivity of AT-PA cells to ionizing radiation.

A linear relationship of the induction of Mn as a function of X-ray doses was observed in both AT-PA and N-SW cell lines at any sampling time. The results at 48 and 72 hours post-irradiation incubation are illustrated in Figure 3. 2. AT-PA cells showed a 2.5 fold and 3.8 fold enhanced yield of Mn at 48 hours and 72 hours post-irradiation incubation, respectively.

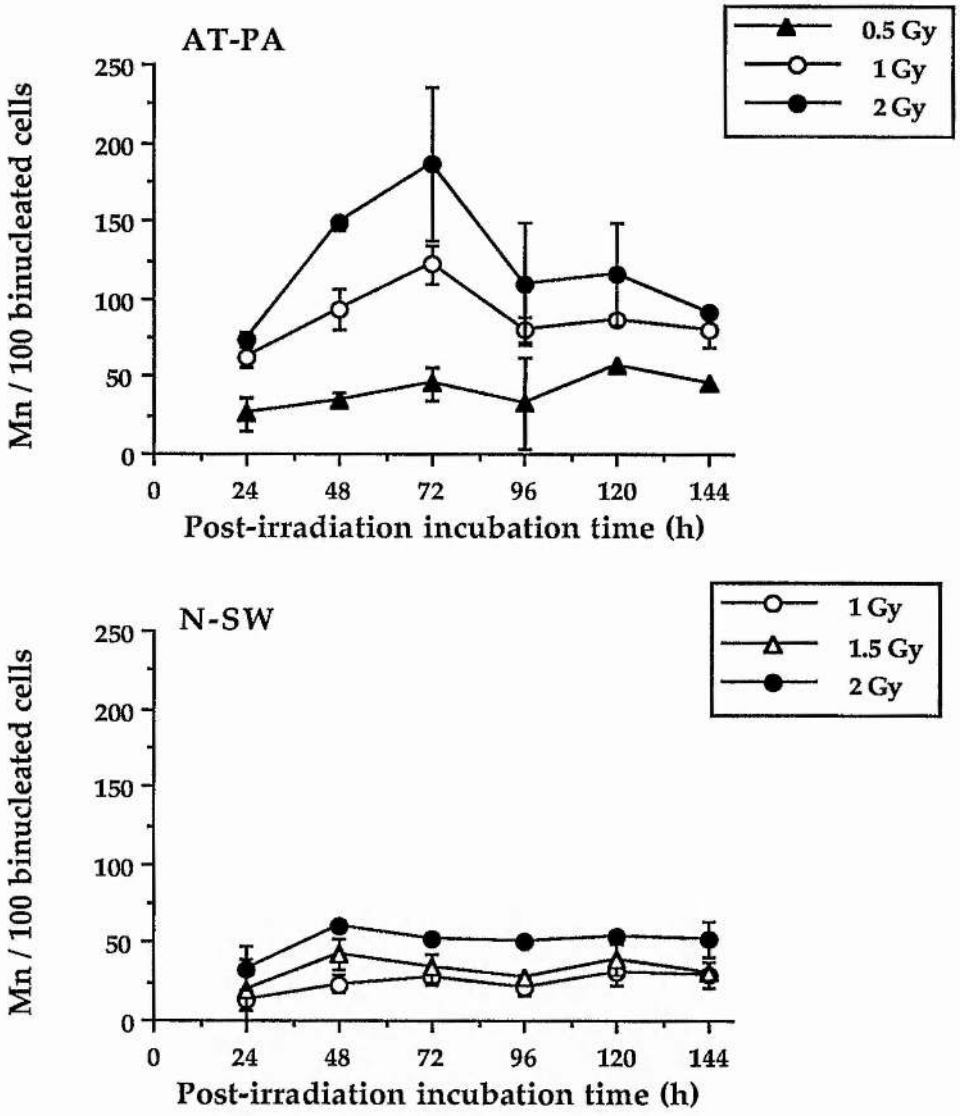

Figure 3. 1. Time-course for the induction of micronuclei (Mn) by X-irradiation in AT-PA (upper panel) and N-SW (lower panel) cells during post-irradiation incubation time for 24 to 144 hours. Error bars represent standard errors of mean values from 3 independent experiments.

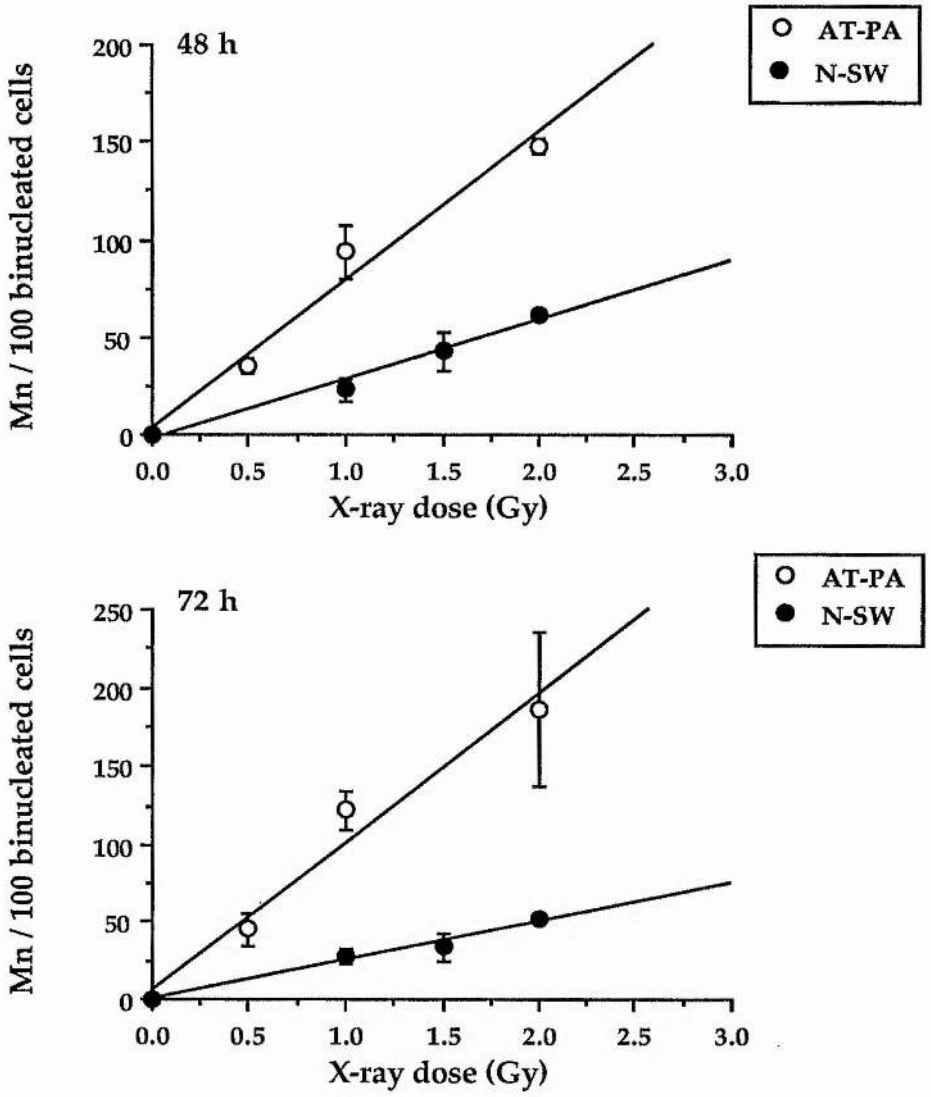

Figure 3. 2. Dose-effect relationships for yields of Mn in X-irradiated AT-PA and N-SW cells at 48 hours (upper panel) and 72 hours (lower panel) incubation after irradiation. Data represent as mean values and standard errors obtained from 3 independent experiments.

The percentage binucleated (BN) cells in the presence of cytochalasin B was determined as a function of post-irradiation incubation time. The results are presented in Figure 3. 3. In unirradiated AT and normal cells, the percentage BN cells decreased with an increase incubation time as cells pass through the cell cycle and divide more than once. There was a marked reduction in the level of BN cells in the normal N-SW cell line 24 hours

after exposure to X-ray, while the extent of this reduction was less in AT-PA cells (Figure 3. 3). These results imply a different of cell cycle progression exists between AT and normal cells in response to irradiation.

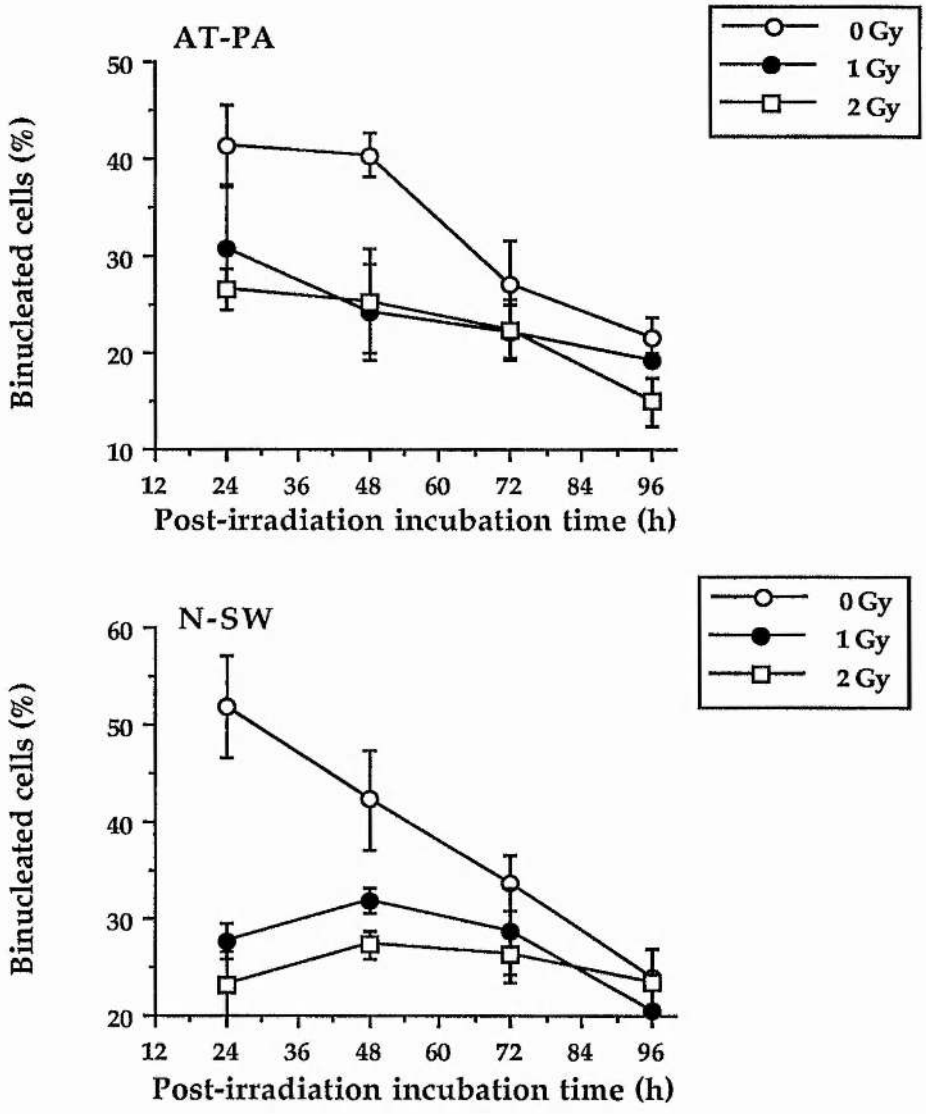

Figure 3. 3. Percentage binucleated cells of the whole cell population of unirradiated and irradiated AT-PA (upper panel) and N-SW cells (lower panel) observed at various incubation times in the presence of cytochalasin B after X-irradiation. Error bars represent standard errors of mean values from 3 independent experiments.

The dose-effect of γ-rays for production of Mn in AT-PA, AT-KM and N-SW cell lines was examined after 48-hour post-irradiation incubation and

a similar linear increase in Mn frequency with increasing γ-ray-dose was observed (Figure 3. 4). Again there were about 3 times more Mn induction by a given γ-ray dose in both AT-KM and AT-PA cell lines than that in the normal N-SW cells.

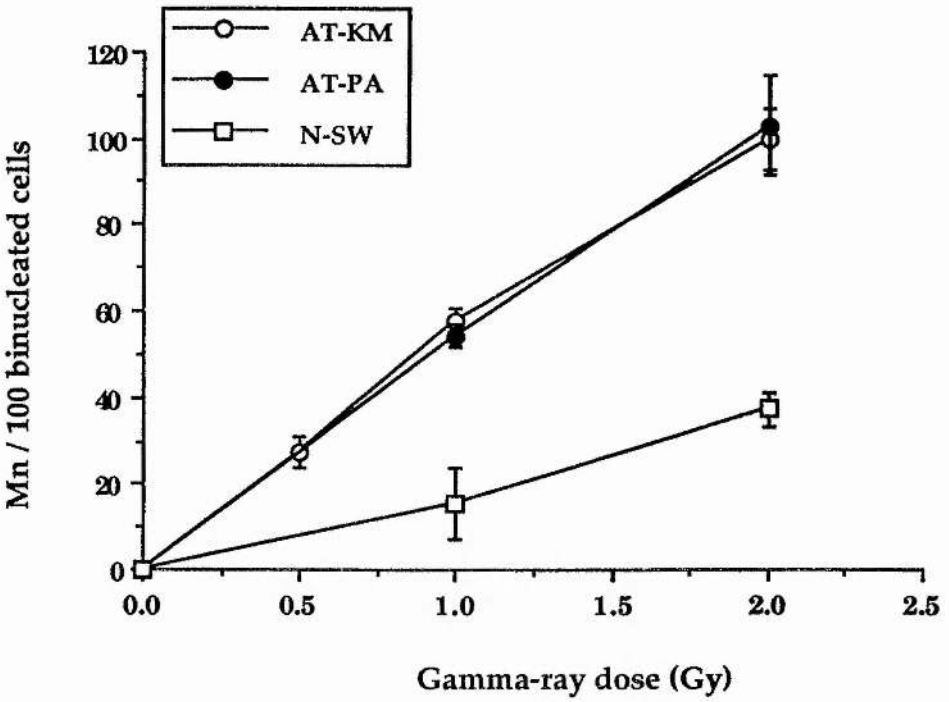

Figure 3. 4. Dose-effect of γ-irradiation on the induction of Mn in AT-PA, AT-KM and N-SW cells after 48 hours post-irradiation incubation. Mean values and standard errors of 3 independent experiments are presented.

3. 2. 2. Chromosomal aberrations induced by γ-irradiation

The induction of chromosomal aberrations in cells irradiated at G_1 or G_2 phase of cell cycle in AT-PA and N-SW cell lines were investigated and the data is summarised in Table 3. 1.

Table 3. 1. Frequencies of chromosomal aberrations (CA) in per AT-PA and N-SW cell γ-irradiated at G_1 (harvested after 30 h) or G_2 (harvested after 3.5 h) phase of cell cycle.

Cell line	γ-ray (Gy)	Incubation time (h)[a]	No. of cells analysed[b]	Chromosome exchange[c]	Chromosome deletion	Chromatid exchange[d]	Chromatid deletion	Chromatid gap	No. of CA per cell	SEM[e]
AT-PA	0	4	100 (1)	0	0	0.01	0.10	0.30	0.41	-
	0.3	3.5	200 (2)	0	0	0	1.95	0.62	2.57	0.02
	0	30	100 (1)	0	0	0	0.09	0.11	0.20	-
	1	30	100 (1)	0.13	0.28	0.04	0.60	0.50	1.55	-
	2	30	200 (2)	0.12	0.33	0.22	1.37	0.47	2.52	0.38
N-SW	0	4	100 (1)	0	0	0	0	0.10	0.10	-
	0.3	3.5	200 (2)	0	0	0	0.47	0.41	0.88	0.19
	0	30	100 (1)	0	0.01	0	0.07	0.12	0.20	-
	1	30	100 (1)	0.11	0.20	0	0.09	0.19	0.59	-
	2	30	200 (2)	0.24	0.24	0.005	0.15	0.32	0.96	0.07

[a]: Time of incubation at 37 °C following irradiation and before collection of the samples.

[b]: Number of independent experiments in parenthesis.

[c]: Including dicentric chromosomes and rings.

[d]: Including asymmetrical or symmetrical quadriradials and triradials.

[e]: Standard errors of mean values of CA per cell.

To examine the chromosomal aberrations resulting from G_1 phase irradiation, cells were incubated for 30 hours after irradiation. To examine aberrations resulting from G_2 phase irradiation, cells were incubated for 3.5 hours before harvesting. A low radiation dose (0.3 Gy) was used for G_2 phase cells because a severe radiation-induced block in the progression of G_2 cells into metaphase by irradiation particularly in N-SW cells has been observed. AT-PA cells showed an increased level of chromosomal aberrations over N-SW cells when the cells were harvested after post-irradiation incubation times of either 3.5 h (cells at the G_2 phase at the time of irradiation) or at 30 h (cells at the G_1 phase at the time of irradiation). When cells were irradiated with 0.3 Gy and sampled after 3.5 h, only chromatid-type chromosomal aberrations were observed in both AT-PA and N-SW cell lines. The frequency of total G_2 phase chromatid aberrations for the AT-PA cell line was approximately 2.8-fold higher than that in the N-SW cell line. When harvested at 30 hours AT-PA cells (exposed to γ-ray in the G_1 phase) exhibited a dramatically increased level of chromatid-type aberrations, particularly chromatid deletions and exchanges, in addition to chromosome-type aberrations. No such increase in chromatid-type aberrations was observed in G_1 N-SW cells. On the other hand, the types of chromosome aberrations observed were not much different between AT and normal cells. The level of chromosomal aberrations in AT-PA cells irradiated in the G1 phase (as selected by incubation for 30 hours post-irradiation incubation) was enhanced by a factor of 3 compared to that in N-SW cells, a result which was consistent with the chromosomal sensitivity of G_2 phase cells and sensitivity measured by the Mn assay.

3. 2. 3. Kinetics of chromosomal aberrations in G_2 phase cells

Colcemid was added to cell culture at 0.5 to 2 hours intervals following 0.3 Gy γ-irradiation and incubation with colcemid carried out for 3 hours, since a short time incubation with colcemid (0.5 h) did not give a sufficient number of cells at metaphase for analysis, probably because of a cell cycle arrest resulting from irradiation. Only chromatid-type aberrations were seen at each time-point tested (Table 3. 2.). Figure 3. 5. illustrates the kinetics of chromatid aberrations in cells irradiated in the G_2 phase for AT-PA, AT-KM and N-SW cell lines. As mentioned above, an elevated frequency of chromatid deletions as well as chromatid gaps appeared in both AT cell lines throughout the incubation, although chromatid exchanges were not always found to be higher in frequency in the AT cell lines compared to the normal cell line (Table 3. 2). A decline in the frequency of aberrations, mainly in chromatid deletions, was observed in each cell lines with increasing incubation time (Figure 3. 5), implying repair of the lesions underlying chromatid aberrations was occurring. While the yield of chromatid aberrations in the two AT cell lines were approximately 3-fold higher than that in the normal cell line, the rate of removal of chromatid aberrations, especially of chromatid deletions by the AT cell lines was similar to the rate observed for N-SW cells.

Table 3. 2. Frequencies of chromatid aberrations (CA) per cell induced by 0.3 Gy γ-irradiation in AT-PA, AT-KM and N-SW cells (3 hours with colcemid at 0.04 μg/ml). Mean values ± standard errors.

Cell line	γ-ray (Gy)	Incubation time (h)[a]	No. of cells analysed[b]	Chromatid exchange[d]	Chromatid deletion	Chromatid gap	Total CA per cell
AT-KM	0	4	100 (1)	0	0	0.18	0.18
	0.3	3.5	200 (2)	0	1.49 ± 0.01	0.98 ± 0.36	2.47 ± 0.37
	0.3	4	200 (2)	0	1.20 ± 0.13	0.63 ± 0.07	1.83 ± 0.06
	0.3	5	200 (2)	0	0.62 ± 0.12	0.56 ± 0.09	1.17 ± 0.21
AT-PA	0	4	100 (1)	0.01	0.10	0.30	0.41
	0.3	3.5	200 (2)	0	1.95 ± 0.12	0.62 ± 0.11	2.57 ± 0.02
	0.3	4	200 (2)	0.025 ± 0.005	1.46 ± 0.15	0.80 ± 0.001	2.29 ± 0.16
	0.3	5	200 (2)	0.015 ± 0.005	1.06 ± 0.06	0.65 ± 0.02	1.73 ± 0.07
N-SW	0	4	100 (1)	0	0	0.10	0.10
	0.3	3.5	200 (2)	0	0.47 ± 0.09	0.41 ± 0.10	0.88 ± 0.19
	0.3	4	200 (2)	0	0.35 ± 0.05	0.34 ± 0.03	0.69 ± 0.02
	0.3	5	200 (2)	0.015 ± 0.01	0.23 ± 0.07	0.29 ± 0.02	0.54 ± 0.07

[a], [b] and [d] have the same meanings as in Table 3. 1.

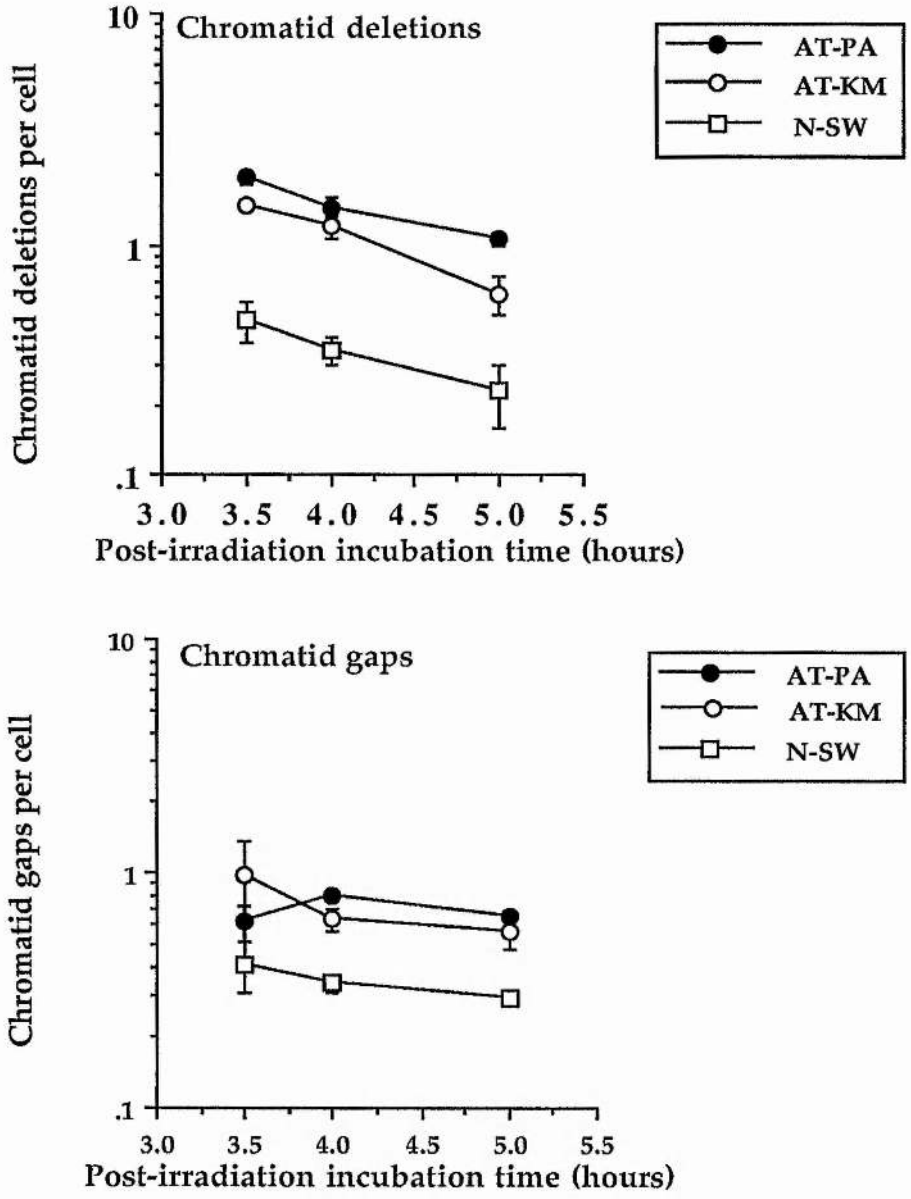

Figure 3. 5. Frequencies of chromatid deletions (upper panel) and of chromatid gaps (lower panel) as a function of post-irradiation incubation time in AT-PA, AT-KM and N-SW cells. Data presented as mean values and standard errors obtained from 2 independent experiments

3. 2. 4. Chromosomal sensitivity to bleomycin

The sensitivity of AT-KM and N-SW cells to bleomycin (BLM) with respect to the induction of chromosomal aberrations was examined. BLM (stock solution: 1 mg/ml in distiled H_2O) was diluted with HBSS and added

to 1 ml of cell suspension (1 x 10^6 cells) at a final concentration of 0.01 μg/ml. Post-treatment incubation was carried out at 37 °C for 4 hours and colcemid added 3 hours prior to fixation. The frequencies of chromatid aberrations induced by BLM are shown in Figure 3. 6. At the low concentration of BLM (0.01μg/ml), N-SW cells were not significantly influenced by the BLM treatment in the production chromosomal damage. However, AT-KM cells showed a marked increase in frequency of chromatid aberrations when compared with N-SW cells. These data indicate a chromosomal instability to BLM exists in the AT cells.

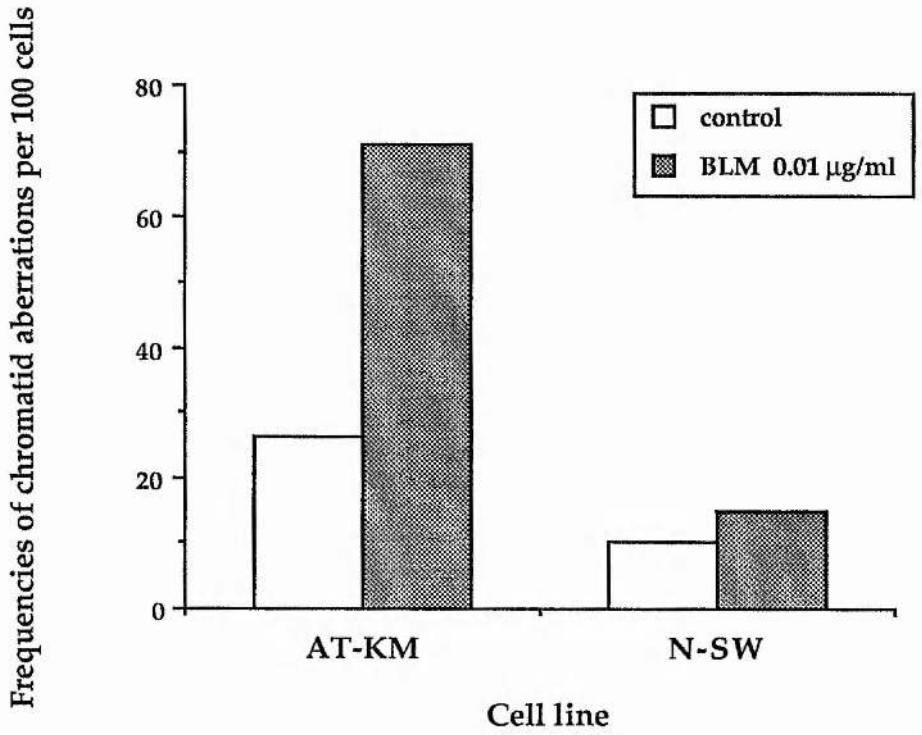

Figure 3. 6. Frequency of chromatid aberrations in AT-KM and N-SW cells exposed to 0.01 μg/ml BLM and and harvested at 4 h post-treatment incubation.

3. 2. 5. Induction and rejoining of double strand breaks in DNA

The induction of dsb by 5-20 Gy of γ-rays measured by neutral filter elution (pH 9.6) in AT-PA and N-SW cell lines is shown in Figure 3. 7. The fraction of DNA eluted is directly proportional to the production of dsb. The

control values were 0.077 ± 0.005 for AT-PA and 0.165 ± 0.029 for N-SW, which values had been subtracted from the data shown in Figure 3. 7. Although the slope of the curve for N-SW cells is higher than that for AT-PA cells, a statistical analysis (student *t*-test) showed no significant difference (except at 10 Gy) between the measurements in AT and normal cells.

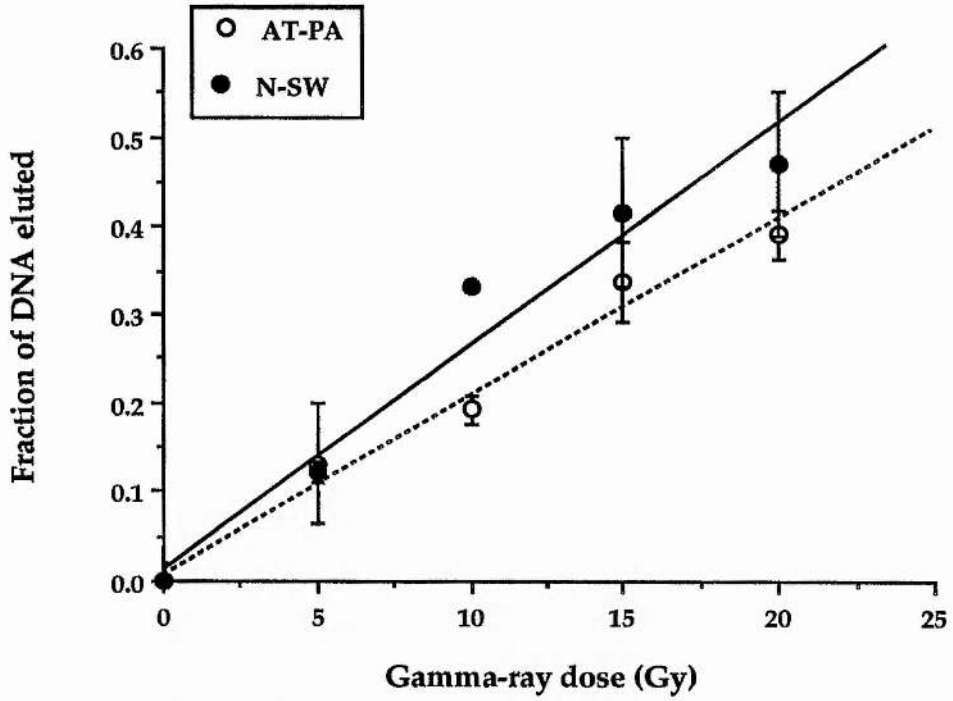

Figure 3. 7. Production of dsb induced by γ-irradiation in AT-PA (broken line) and N-SW cells (solid line) as measured by the fraction of DNA eluted using neutral filter elution at pH 9.6. Curves were fitted by compter. For AT-PA: $y = 0.0126 + 0.01976x$ ($R^2 = 0.981$); For N-SW: $y = 0.0208 + 0.0247x$ ($R^2 = 0.954$).

During post-irradiation incubation at 37 °C after 20 Gy irradiation, cells of both the AT and normal cell lines were capable of rejoining dsb. The rejoining rate of dsb for AT-PA cells was found to be similar to that of normal N-SW cells (Figure 3. 8), although the average values of ratio of dsb rejoined for AT-PA cells were lower (but not significantly) than those for N-SW cells at each time-point. The rejoining of dsb seemed to be complete at 120 min of post-incubation for both AT-PA and N-SW cell lines.

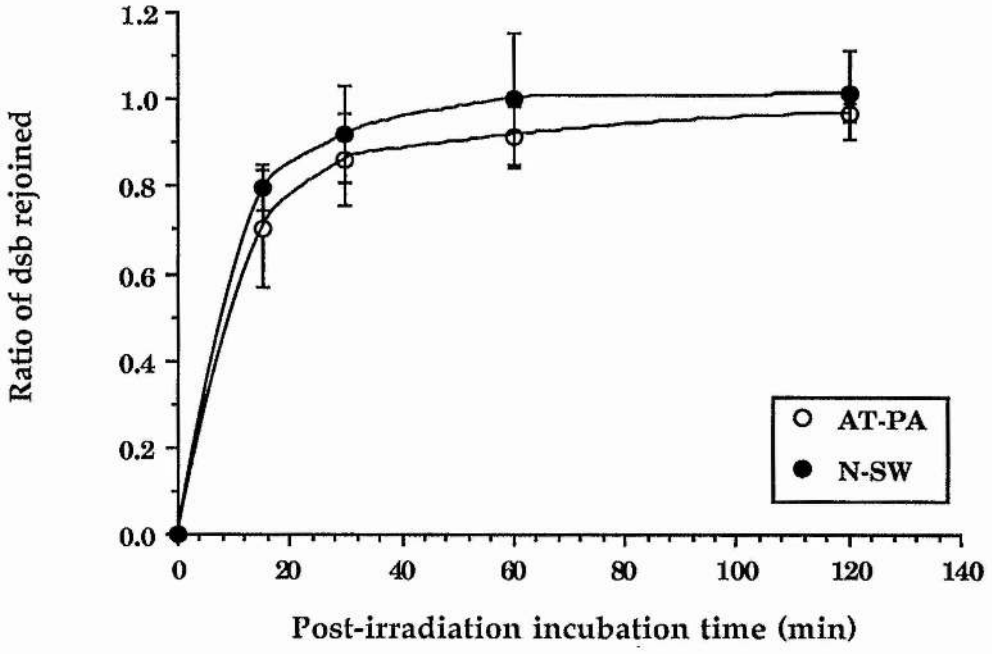

Figure 3. 8. Rejoining of dsb in AT-PA and N-SW cells exposed to 20 Gy γ-rays. The ratio of dsb rejoined was calculated by the formula described in Chapter 2, section 2. 9. Data were pooled from at least 3 independent measurements and the standard errors are shown.

3. 2. 6. Cell cycle response

The proportion of cells at different stages of the cell cycle was examined using a flow cytometric technique. Irradiation of cells results in a cell cycle perturbation which occurs mainly at G_2 phase and leads to an accumulation of cells at G_2 phase. The accumulation of G_2 phase cells exhibits a radiation-dose-related increase and increases with incubation time. An example of the dose-related and time-related alteration of cell cycle progression of N-SW cells is presented in Figure 3. 9. and Figure 3. 10. A significant accumulation of S phase cells was also detected in N-SW cells 6 h after irradiation (Figure 3. 10), although this phenomenon was not observed in AT-PA and AT-KM cells (data are not shown).

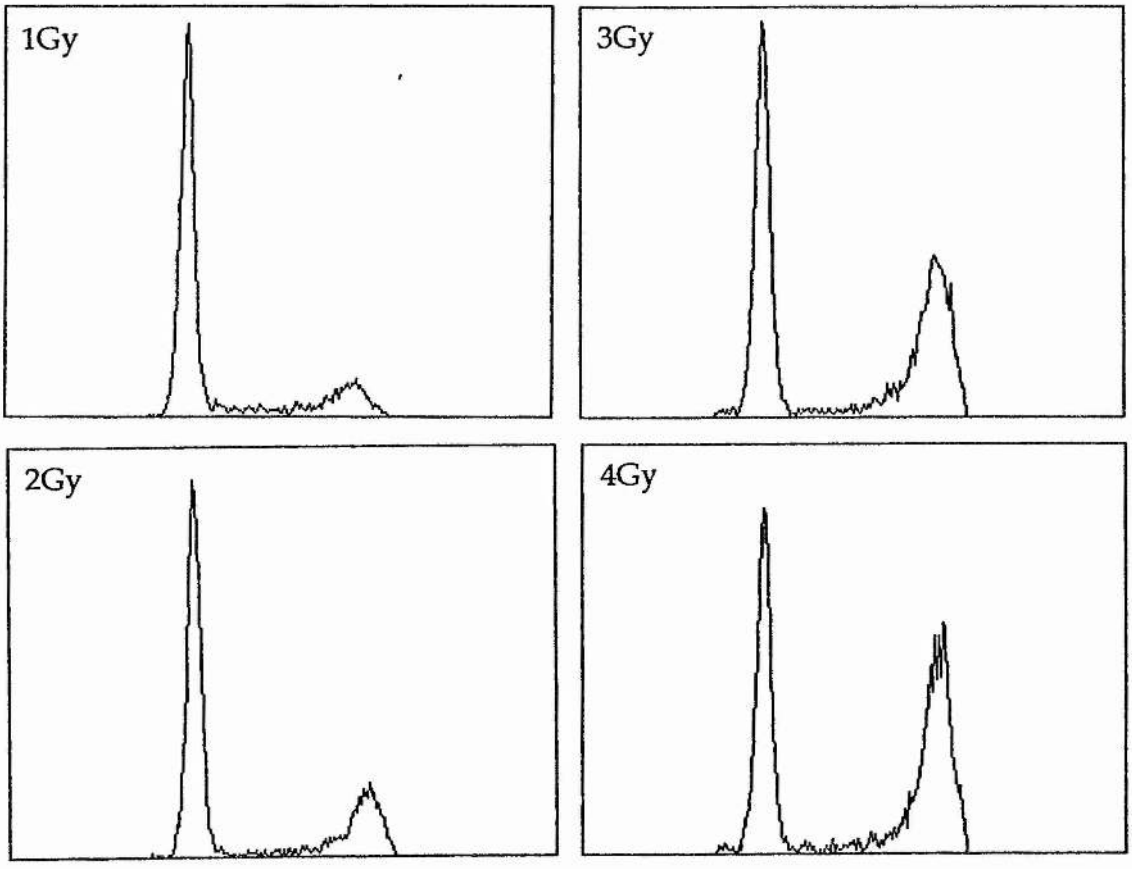

Figure 3. 9. Profile of N-SW cell populations at different cell cycle stages after various doses of γ-irradiation and incubated for 24 hours post-irradiation.

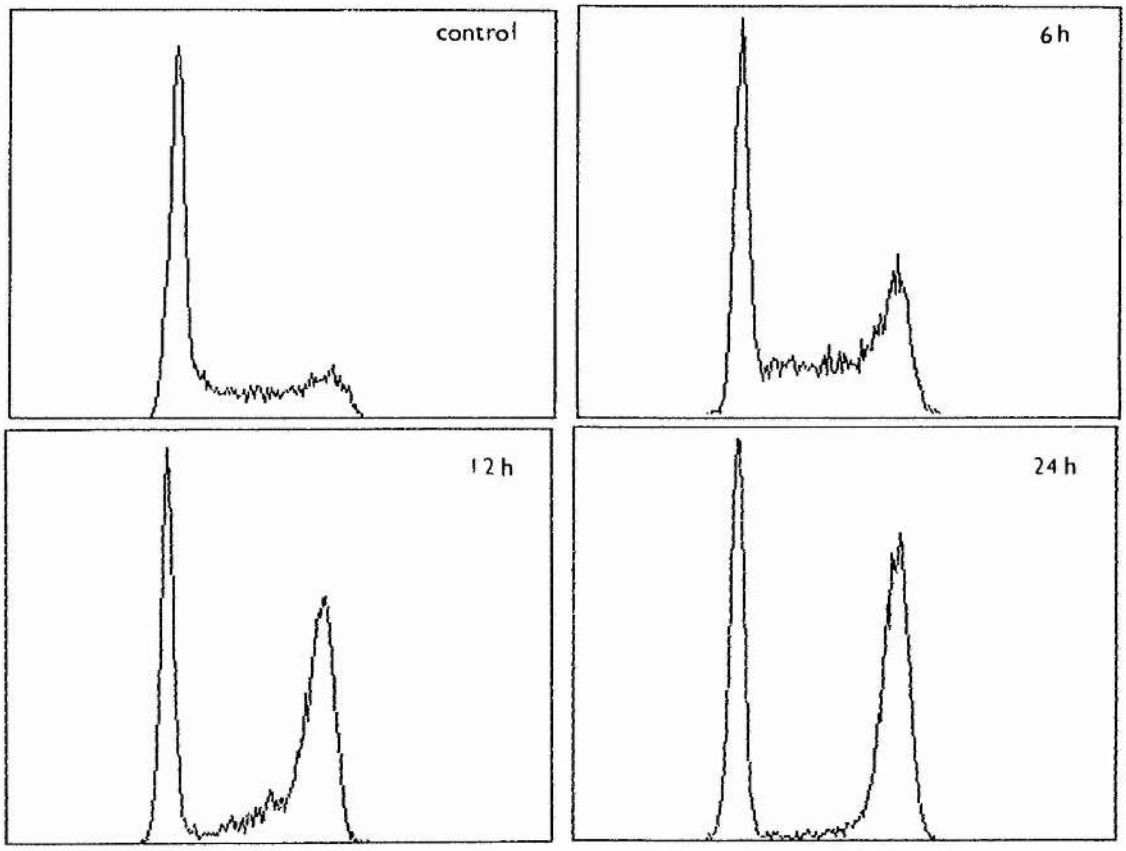

Figure 3. 10. Profile of N-SW cell populations at different cell cycle stages after 4 Gy of γ-irradiation and post-irradiation incubated for various times.

Figure 3. 11. shows the populations of AT-PA, AT-KM and N-SW cells at G_2/M phase which were irradiated with various doses of γ-rays and harvested 24 hours after irradiation. The data shows that while AT-KM cells exhibit an increased accumulation of cells at G_2/M phase when compared with N-SW cells, AT-PA cells showed no significant difference from N-SW cells.

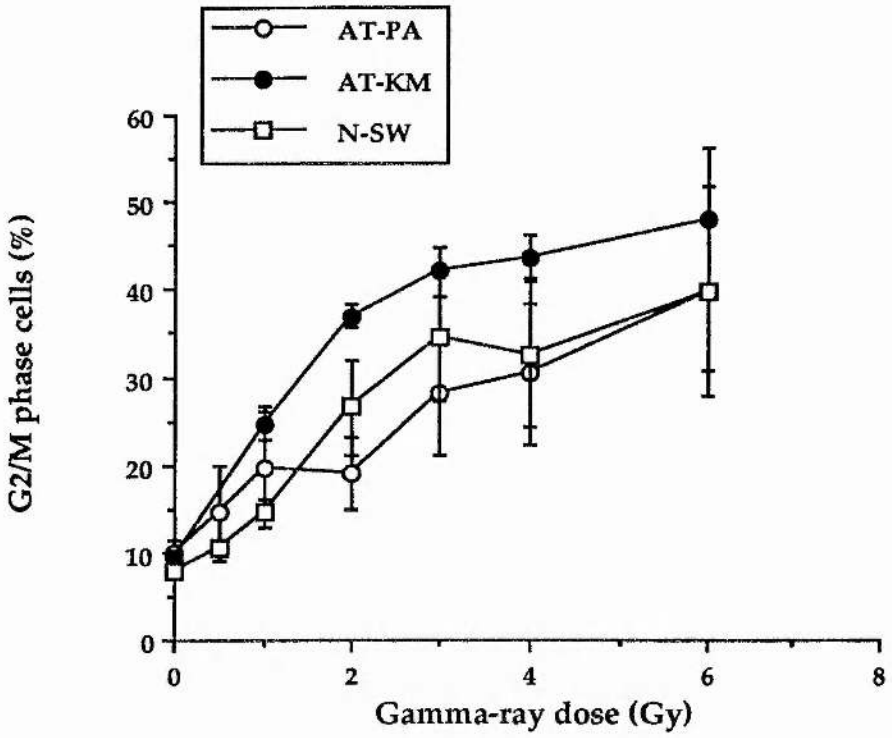

Figure 3. 11. Percentage G_2/M phase cells in AT-PA, AT-KM and N-SW cell lines as a function of γ-irradiation dose. The cells were incubated for 24 hours at 37 °C following irradiation and the cell population was determined by flow cytometry. Data were obtained from 3 (for AT-PA and AT-KM cell lines) and 4 (for N-SW cell line) independent experiments and the mean values and standard errors are presented.

3. 2. 7. DNA Synthesis

The effects of X- or γ-irradiation on DNA replication in AT-PA and N-SW cells were investigated by the rate of incorporation ^{3}H-TdR into newly synthesised DNA. AT-PA cells exhibited a reduced inhibition of DNA

synthesis following irradiation when compared with N-SW cells. These results are presented in Chapter 6.

3. 3. Discussion

Chromosomal sensitivity of AT-PA and AT-KM lymphoblastoid cell lines to ionizing radiation

AT-PA and AT-KM cell lines exhibited increased chromosomal radiosensitivity after exposure to ionizing radiation compared with the normal N-SW cell line, as measured by the production of micronuclei and of chromosomal aberrations in cells irradiated at either the G_1 or G_2 phases of cell cycle. The frequency of Mn in X-irradiated AT-PA cells was consistently higher than that in normal N-SW cells by factors ranging from 2.4 (24 h) to 3.8 (72 h) during the period of post-irradiation incubation. The highest frequency of Mn in AT cells was observed after 72 h post-irradiation incubation (Figure 3. 1). The increased frequencies of Mn in AT-PA cells are consistent with elevated levels of chromosomal aberrations in AT-PA which showed 2.8 and 3.5 times higher than in N-SW following irradiation at G_1 (sampled after 30 hours) and G_2 phase (sampled after 4 hours), respectively. These data also agree well with the enhanced cell killing effects of irradiation on AT cells which have been reported to be in the range of 2 to 4 (Taylor et al 1975, Arlett and Harcourt 1980). A higher chromosomal sensitivity to bleomycin was also observed in AT-KM cells when compared with normal human cells (Figure 3. 6). These results indicate an intrinsic chromosomal hypersensitivity of AT-KM and AT-PA cell lines to ionizing radiation and the radiomimic agent, characteristics generally found in AT cells (Rary et al 1974, Taylor 1978, Bender et al 1982, Natarajan and Meyers 1979, Mozdarani and Bryant 1989a).

After exposure to radiation and post-irradiation incubation for 30 hours, AT-PA cells (irradiated at G_1 phase) showed the effect observed in lymphocytes and other primary cell lines from AT patients (Taylor 1978) namely an increased frequency of chromatid-type aberrations which was not found in normal cells (Table 3. 1). Chromatid exchanges were also found at a higher frequency in G_1 phase irradiated AT-PA cells (Table 3. 1) but not always found in G_2 phase irradiated AT cells (Table 3. 2). However, the chromosome-type aberrations seemed to be not very different between AT and normal cells when cells were irradiated in the G_1 phase (Table 3. 1). Both the AT and normal cells in the G_2 phase yielded more chromosomal aberrations than cells in G_1 phase after exposure to irradiation. Similar results have been reported by Taylor (1978), and the lower frequencies of G_1 aberrations has been suggested to be due to the repair of dsb during DNA replication and due to a lack of progress of heavily damaged or unrepaired cells through the cell cycle.

The extent of the enhanced level of chromosomal aberrations observed in the transformed AT cell lines used was less marked than those in primary AT cell lines and lymphocytes derived from AT individuals (Taylor 1979, Natarajan et al 1978), but is close to that reported for transformed AT fibroblast cell lines (Mozdarani and Bryant 1989b). The difference between primary and transformed cell lines may be the result of the selection of a subgroup of cells during the establishment of the cell line.

Kinetics of G_2 chromosomal damage

The kinetics of chromatid aberrations in G_2 phase irradiated cells showed a reduction in frequency with increasing post-irradiation incubation times (Figure 3. 5). This was observed to be similar in the two AT and normal cell lines. The rate of disappearance of total chromatid aberrations, especially of chromatid deletions, were not significantly different between

AT and normal cell lines. The half times ($t_{1/2}$) for the disappearance of G_2 chormosomal damage were estimated to be 3.4, 3.1 and 3.5 hours for AT-PA, AT-KM and N-SW cells, respectively, following irradiation at 0.3 Gy. These values are similar to those obtained with G_2 assay for fibroblasts, which are 3.1 and 2.7 hours for AT5BIVA and for MRC5SVI cell line, respectively (Mozdarani and Bryant 1989b).

Since the kinetics of chromatid breaks in present studies were found to be the same as those obtained for human fibroblasts (Mozdarani and Bryant 1989a) although a longer time of incubation with colcemid (3 hours) was used, it is possible that the initial level of chromatid aberrations can still be obtained by extrapolating the curves of chromatid aberrations (Figure 3. 5) back to "zero time". The average numbers of chromatid breaks per cell induced by 1 Gy of radiation so obtained were approximately 12 (26 per 100 chromosomes) for both AT-PA and AT-KM lines and 3 (7 per 100 chromosomes) for the normal cell line, which were comparable to the data so deduced by Mozdarani and Bryant (1989b) who have reported 22 and 9 chromatid breaks per Gy per 100 chromosomes of AT and normal cells, respectively.

The disappearance of chromatid aberrations may be a reflection of repair of DNA damage in G_2 phase. It has been demonstrated in synchronised mammalian cells that both ssb (Graubmann and Dikomey 1983) and dsb (Blöcher et al 1983) are rejoined in G_2 phase at similar rates as in G_1 and S phases. Although the rates of repair of chromosomal damage were apparently the same for the AT and normal cells, the overall yields of aberrations in AT-PA and AT-KM cell lines were consistently higher than those in normal cells by factors of 2.8 and 2.5, respectively. These observations are in agreement with those reported by Mozdarani and Bryant (1989a). Similar results have also been found in dsb repair-deficient *xrs*-5 cells, which show an elevated frequency of chromatid aberrations induced

by γ-irradiation when compared to its wild-type CHO cells, while the chromosomal repair kinetics in *xrs*-5 cells were not different from those in CHO cells following G_2 phase irradiation (MacLeod and Bryant 1990).

The implication of these findings may be that the repair of chromosomal damage in the G_2 cells cannot be a sole reflection of the capability of cells to repair dsb. The consistently higher number of chromatid aberrations during the disappearance of chromosomal damages in AT cells may result from a specific class of DNA lesions that is prone to be unrejoined or misrejoined, while another class of reparable lesions may represent a component of the portion of chromatid aberrations which decrease in frequency with incubation time. The existence of two components, namely reparable and irreparable of dsb (Dahm-Daphi et al 1993) and PCC breaks (Iliakis and Pantelias 1990), has been demonstrated in *xrs*-5 cells. The kinetics of rejoining of reparable dsb in *xrs*-5 cells have been found not to differ from those of CHO cells, but the level of irreparable dsb is higher in these mutant cells by a factor of 3 - 4 when compared with CHO cells (Dahm-Daphi et al 1993). Although AT cells are different from the *xrs* mutants in that AT cells generally show a normal rejoining of dsb while *xrs* cells are deficient in dsb repair, PCC experiments with AT or *xrs*-5 cells have found a similarly higher induction of PCC breaks and higher level of residual PCC breaks in both AT and the *xrs* mutant. It is plausible to assume that the level of irreparable DNA lesions, which lead to the increased frequency of chromatid aberrations, is also higher in AT cells than in normal cells, although this may be caused by a different mechanism from that in *xrs* mutant because the rejoining of dsb in AT cells occurs apparently normally.

The relationship between production and repair of dsb and chromosomal aberrations in AT cells.

As has been reported for many AT cell lines, the induction of dsb immediately following 5 - 20 Gy of γ-irradiation was found not to be significantly different between AT-PA and N-SW cells (Figure 3. 7). The rejoining of dsb induced by 20 Gy irradiation was rapid and complete after 1 to 2 hours of post-irradiation incubation for both AT-PA and N-SW cells (Figure 3. 8). The rate and extent of rejoining of dsb in AT-PA cells was apparently normal. The half times ($t_{1/2}$) of dsb rejoining were 8 - 10 min and the repair completed in incubation intervals of 60 - 120 min for both AT-PA and N-SW cell lines. The kinetics of dsb rejoining were similar to the results obtained using neutral filter elution (Weibezahn and Coquerelle 1981). Iliakis et al (1991) investigated the initial rate of dsb rejoining in plateau-phase CHO cells by means of several major techniques currently used to quantify dsb, including non-unwinding filter elution, neutral sucrose gradient centrifugation and two pulsed-field gel electrophoresis techniques (PFGE) (asymmetric field inversion gel electrophoresis and clamped homogeneous electric field gel electrophoresis). These authors observed similar values for the initial rate of dsb rejoining with all assays used, with $t_{1/2}$ 10 - 12 min after exposure to 25 Gy and 15 - 20 min after exposure to 50 Gy. Therefore, $t_{1/2}$ (8-10 min after 20 Gy irradiation) obtained in present experiment were in good agreement with their observations. However, these values are much lower than those obtained by other authors, e.g. 1 h in human fibroblasts using the PFGE technique (Blöcher et al 1991) and 1.5 - 4 h in mouse tumour cells using the neutral sucrose gradient centrifugation (Bryant and Blöcher 1980). These $t_{1/2}$ values are similar to $t_{1/2}$ values for chromatid breaks rejoining in G_2 cells observed in this study and those reported by Mozdarani and Bryant (1989b), and those for rejoining of PCC fragment (Cornforth and Bedforth 1983, Iliakis et al 1991).

The fact that the production of chromosomal damage is always much lower than that of dsb indicates that chromosomal aberrations arise from a small portion of dsb, which may account for 10 - 15% of the total initial dsb induced by irradiation as has been speculated by Taylor (1979) and Cornforth and Bedford (1983). This component of dsb may be rejoined more slowly and subsequently be expressed as PCC breaks or chromosomal aberrations. One hypothesis to explain this may be that dsb induced in certain regions of chromatin are more difficult to repair than those in other regions. This fraction of dsb may be higher in AT cells possibly as a result of increased frequencies of these susceptible sites in the chromatin. It has been suggested that AT cells possess abnormal chromatin structure and the response of loop supercoiling to ionizing radiation (as reviewed in Chapter 1).

The extent of dsb rejoining after 1 - 2 hours incubation is apparently similar in AT-PA and N-SW cells. It should be noted that the ratio of dsb rejoined at various post-irradiation incubation times were consistently lower in AT cells than in the normal cells, although the differences were not statistically significant partially because of the large intrinsic variations in the measurements. The average fraction of residual dsb was found to be 4 - 8% in AT-PA while in normal cells dsb seemed to be completely rejoined by 1 hour incubation. These values for residual dsb are similar to those found by Blöcher et al (1991) who used the PFGE technique and demonstrated a higher fraction of residual dsb in AT cells (5.2%) than that in normal cells (1.5%). The values appear to be too low to be significant since the general error of the methods employed would be as high as 10%. However, on the assumption that these values reflect a lower ability of AT cells to completely rejoin dsb, they do correlate with the higher initial frequencies of chromosomal aberrations observed in AT cells in comparison to normal cells (Mozdarani and Bryant 1989a, Pandita and Hittleman 1992). On the basis of studies of chromosomal aberrations, Taylor (1989) proposed

that following G_2 irradiation the number of unrepaired dsb detected cytogenetically in AT cells may be about 5 - 10% and more than 90% of repair is complete by 4 hours. Whereas by this time, greater than 99% of dsb are repaired in normal cells with only 1% remaining unrepaired.

Thus, the observed "normal" rejoining of bulk dsb in AT cells cannot represent a functional repair of dsb, therefore chromosomal repair may be a more reliable guide of the ability of AT cells to repair the lesions induced by ionizing radiation. The lesions which lead to an elevated frequency of chromosomal aberrations in AT cells are probably a specific class of dsb but they may not be detectable by current techniques.

Cell cycle response

The block in cell cycle progression in response to irradiation has been postulated to increase the time available for repair before damage to DNA becomes "fixed" (Painter and Young 1980). In spite of a similarly increased clastogenicity after irradiation and the similar chromosome repair kinetics in AT-PA and AT-KM cell lines, a higher proportion of cells accumulating at G_2 phase was found in AT-KM cells but not in AT-PA cells after exposure to various doses of γ-irradiation, when compared with normal cells (Figure 3. 11). If the G_2 delay plays a key role in the determination of chromosomal sensitivity, one would predict a marked difference in the G_2 delay between AT and normal cells. However, AT-PA cells showed no consistent significant difference from normal cells in radiation-induced G_2 arrest. These results indicate that the cell cycle perturbation is not critically related to chromosomal sensitivity and is therefore probably not related to cellular radiosensitivity. The lack of correlation between radiation-induced G_2 arrest and increased cell killing has been reported in CHO and human tumour lines based on studies of cell cycle-blocking modulators (Rowley and Kort 1988, Musk 1991).

A reduced inhibition of DNA synthesis was observed in AT-PA cells compared with N-SW cells following ionizing radiation. These results will be discussed in Chapter 6.

In conclusion, AT-PA and AT-KM cell lines used in this study are hypersensitive to ionizing radiation and are also hypersensitive to bleomycin (as observed in AT-KM cells), radiation resistance of DNA synthesis (as observed in AT-PA cells) and elevated accumulations of G_2 phase cells induced by ionizing radiation in one (AT-KM) out of two AT cell lines. The kinetics of rejoining of dsb (in AT-PA cells) as well as repair of G_2 phase chromatid aberrations (in AT-KM and AT-PA cells) were not found to be different when compared with the normal N-SW cells. A small fraction of undetectable residual dsb and incorrectly rejoined dsb are possibly responsible for the increased frequency of chromosomal aberrations in AT cells.

Chapter IV

Cytogenetic Responses of AT and Normal Cells to Double-strand Breaks Induced by Restriction Endonucleases

4. 1. Introduction

4. 2. Results

4. 2. 1. Induction of micronuclei by treatment with *Pvu* II in SLO porated AT-PA and N-SW cells

4. 2. 2. Induction of chromosomal aberrations by *Pvu* II in AT-PA and N-SW cells

4. 2. 3. Effects of SLO concentration on the induction of chromosomal aberrations by *Pvu* II in AT-PA and N-SW cells

4. 2. 4. Induction of chromosomal aberrations by *Bam*H I in AT-PA and N-SW cells

4. 2. 5. Comparison of chromosomal sensitivity of AT and normal cells to RE producing dsb with blunt- or cohesive-termini

4. 2. 6. Production of dsb in DNA of cells by RE treatment

4. 2. 7. Cell poration assay

4. 2. 8. Assay of stability and activity of RE *in vitro*

4. 3. Discussion

4. 1. Introduction

The hypersensitivity of AT cells to ionizing radiation has been postulated to result from an intrinsic defect in the repair or processing of DNA lesions. To date the nature of these lesions remains unclear. Among various lesions induced by ionizing radiation in DNA, double-strand breaks are generally regarded as the critical lesions that are responsible for cell killing, chromosomal aberrations and other cellular endpoints. AT cells have been assumed to be particularly sensitive to DNA breakage, however, the biological consequences of dsb induced by ionizing radiation have proved difficult to evaluate since irradiation induces various types of lesions in DNA amongst which dsb are only a small component. Studies using plasmid DNA have shown that AT cells are more likely than normal cells illegimately to rejoin restriction endonuclease cut strands of plasmids (Cox et al 1984, Debenham et al 1987, North et al 1990, Miyajima et al 1993, Powell et al 1993). These findings suggest a deficient dsb repair process exists in AT cells.

Restriction endonucleases that exclusively produce dsb in chromatin DNA of permeablized and porated cells have proved to be a useful tool with which to analyze the nature of the lesions responsible for the induction of chromosomal aberrations (Bryant 1984, Natarajan and Obe 1984). Studies of the cytotoxicity of RE have shown that RE mimic the effect of radiation not only on chromosomal aberrations (Bryant 1984, Natarajan and Obe 1984, Obe et al 1986a), but also on cell survival (Bryant 1985, Giaccia et al 1990), mutations (Obe et al 1986b, Singh and Bryant 1991) and oncogenic transformations (Bryant and Riches 1989). The end-structures of RE-induced dsb, i.e., blunt or staggered ends, are thought to determine the different cytotoxicities and cytogenecity of RE; those causing blunt-ended dsb are

potentially more effective than those causing cohesive-ended dsb (Bryant 1984, Natarajan and Obe 1984, Bryant and Christie 1989, Moses et al 1990).

Investigations of the effectiveness of RE on radiosensitive mutant cell lines have shown that most of the Chinese hamster mutant lines, as well as cells from *scid* mice, are more sensitive to RE treatment than wild-type cell lines, showing increased yields of chromosomal aberrations and reduced clonogenicity (Bryant et al 1987, Dorroudi and Natarajan 1989, Cortés and Ortiz 1991, Giaccia et al 1990, Bryant et al 1993, Chang et al 1993).

The mutant cell lines of the *xrs* group, together with XR-1 and *scid* cells, appear to be radiosensitive as a result of a deficiency in the repair of dsb (Kemp et al 1984, Giaccia et al 1985, Biedermann et al 1991). Other mutants such as the *irs* group (*irs*-1, 2 and 3) (Jones et al 1987) and V-group (V-C4, V-E5 and V-G8) (Zdzienicka et al 1989) show apparently normal kinetics of repair of dsb induced by ionizing radiation but manifest 2 - 3 fold elevated sensitivities over corresponding wild type lines. From this point of view, *irs* and V-group mutants appear to resemble AT cells. Furthermore, each *irs* cell line seems to share some but not all of the characteristics of AT cells following irradiation, e.g., resistant DNA synthesis (*irs*-2), prolonged G_2 delay (*irs*-2) and the occurrence of a high frequency of chromatid aberrations after G_1 irradiation (*irs*-1) (Jones et al 1990, Cheong et al 1992).

Therefore, the greater sensitivities of *xrs*-5, XR-1 and *scid* mouse cells to RE may be attributed to a reduced capacity in the repair of dsb, but this cannot account for the hypersensitivity of *irs*-2 cells to RE. The *irs*-2 line has been shown to be chromosomally hypersensitive to *Pvu* II, displaying a 2 - 4 fold increased yield of chromosomal aberrations over that in the parental V79 cells (Bryant et al 1993). In contrast, V-C4 and V-G8 cell lines, although they belong to the same complementation group as *irs*-2 (Zdzienicka et al 1989, Thacker and Wilkinson 1991) and share many similarities in their response to ionizing radiation with AT cells (Zdzienicka et al 1989), have

been reported to show a normal sensitivity to RE treatment (Natarajan et al 1993). These results were explained as that the increased chromosomal sensitivities to DNA damage in these mutant cell lines are not due to a defect in dsb processing but by some different mechanisms (Natarajan et al 1993). However, recent experiments in our laboratory have shown G_2 VC-4 cells to be more sensitive to blunt-ended dsb caused by *Pvu* II than V79 cells in the induction of chromosomal aberrations (Bryant 1994 manuscript in preparation).

It was therefore important to investigate AT cells with respect to their sensitivity to RE-induced dsb to reveal the proposed defect in the repair of dsb at a chromosomal level and to elucidate the mechanism of the chromosomal instability of AT cells. This chapter concerns experiments in which the response of AT and normal lymphoblastoid cells to RE treatment were investigated. The introduction of RE into cells was achieved by SLO poration. The restriction enzymes used were either those causing blunt-ended (*Pvu* II and *Eco*R V) or those causing 5'- (*Eco*R I and *Bam*H I) and 3'-overhang (*Pst* I) cohesive-ended dsb. These enzymes were chosen for their similar cutting frequencies in genomic DNA (Table 2. 1).

4. 2. Results

4. 2. 1. Induction of micronuclei by the treatment of *Pvu* II in SLO porated AT-PA and N-SW cells

The production of micronuclei (Mn) by *Pvu* II in cells porated with various concentrations of SLO was examined in order to optimize the concentration of SLO for porating human cells. Cells were treated with SLO in the concentration range 0.02 - 0.14 units/ml in the absence or presence of *Pvu* II (200 units/ml), by the method described in Chapter 2 (section 2. 4),

followed by an incubation in medium containing CYT-B (3 μg/ml) for 48 hours. Mn were scored in binucleated cells.

Figure 4. 1 shows the results of these experiments for AT-PA and N-SW cells. The frequencies of Mn induced by *Pvu* II exhibited biphasic kinetics with increasing SLO concentrations in both AT-PA and N-SW cell lines. Since an increased background yield of Mn (the yield of Mn in the absence of RE) and a reduced percentage of binucleated cells (data not shown) was observed at high concentration of SLO (greater than 1.0 unit/ml), an optimal concentration of 0.06 units/ml was adopted, and was subsequently used for the standard assay.

Comparing the frequencies of Mn observed at 200 units/ml of *Pvu* II, higher levels of Mn were observed in N-SW cells in comparison to those in AT-PA cells (Figure 4. 1). AT-PA cells showed an abnormally high level of background Mn before or after treatment of SLO in the absence of *Pvu* II.

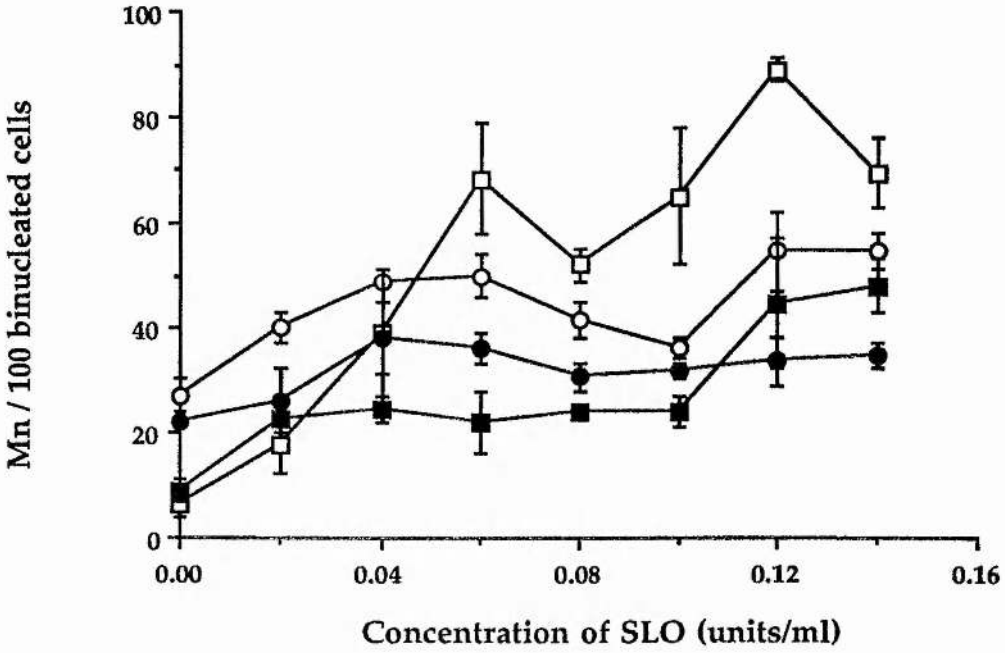

Figure 4. 1. Frequencies of micronuclei in AT-PA (circle) and N-SW (square) cells treated with various concentrations of SLO in the absence (solid symbols) or presence (open symbols) of 200 units/ml *Pvu* II. Mean values and standard errors obtained from 2 independent experiments are presented.

Figure 4. 2 shows the frequencies of Mn as a function of concentration of *Pvu* II and *Eco*R I in cells porated with 0.06 units/ml of SLO and harvested 48 hours after treatment. The results show that *Pvu* II treatment results in higher numbers of Mn than *Eco*R I in both AT and normal cells. The absolute frequency of Mn in AT-PA cells was less than that in N-SW cells when the high level of background Mn in the AT cells was subtracted.

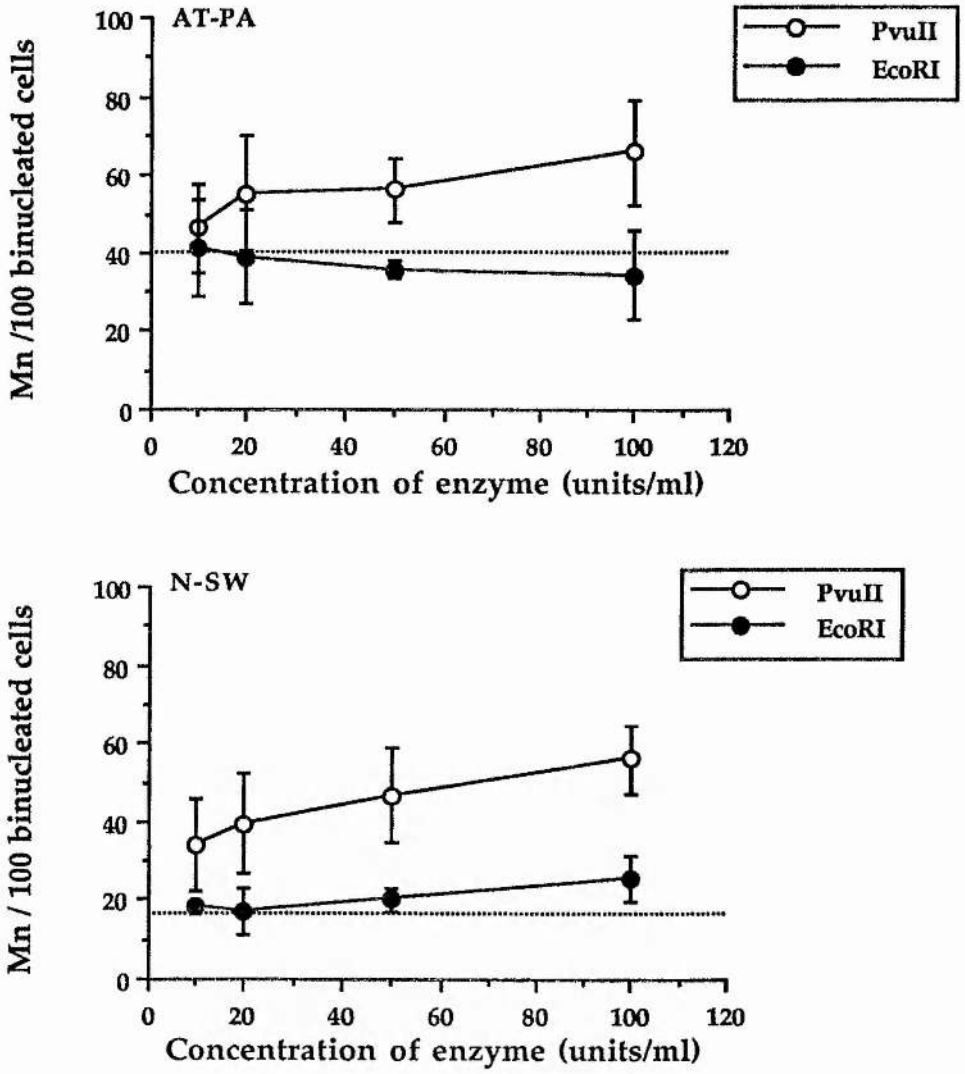

Figure 4. 2. Frequencies of micronuclei as a function of *Pvu* II and *Eco*R I concentrations in AT-PA (upper panel) and N-SW (lower panel) cells. Dotted lines represent the levels of background Mn. The cells were porated with 0.06 units/ml of SLO and harvested 48 hours after treatment. Vertical bars represent standard errors of mean values derived from 3-5 independent experiments.

4. 2. 2. Induction of chromosomal aberrations by the *Pvu* II in AT-PA and N-SW cells

Chromosomal aberrations induced by Pvu II

By the use of the poration technique employing SLO, the restriction endonuclease *Pvu* II (purified as described in Chapter 2) was introduced into human lymphoblastoid cells and the induction of chromosomal aberrations investigated. All the various types of chromosomal aberrations seen following ionizing radiation were observed after RE treatment. Figure 4. 3 illustrates examples of aberrant metaphases containing dicentric or polycentric chromosomes and fragments (Figure 4. 3a), chromatid deletions and gaps (Figure 4. 3b), chromatid exchanges (quadriradials) (Figure 4. 3c) and complicated damage involving chromatid exchanges and deletions (Figure 4. 3d).

It was observed that the polycentric chromosomes (as shown in Figure 4. 3a) were more typically found in N-SW cells, while the complicated damaged metaphase (as shown in Figure 4. 3d) were more typically found in AT-PA cells.

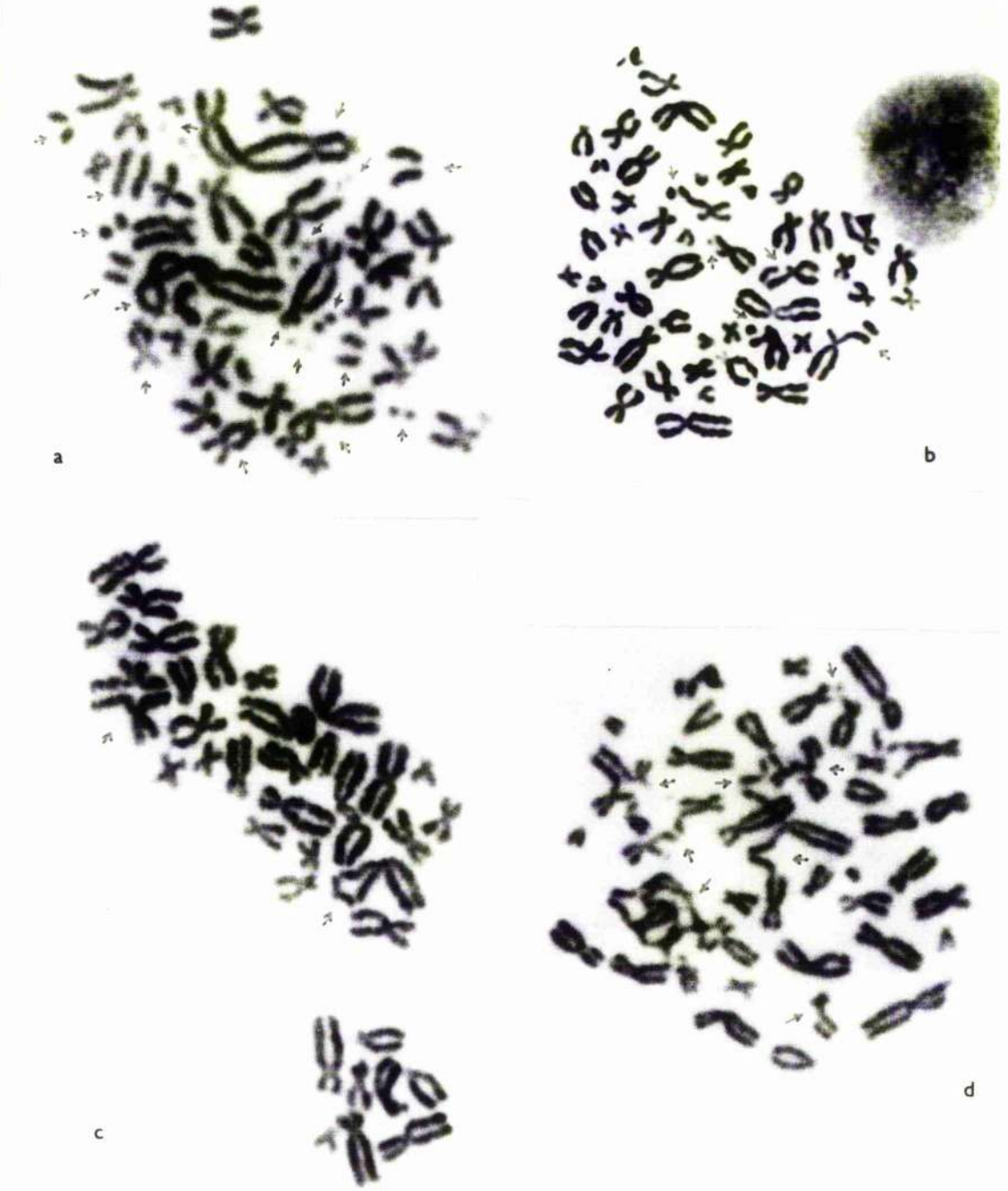

Figure 4. 3. Illustrations of aberrant metaphase cells resulting from treatment with *Pvu* II. a: dicentric or polycentric chromosomes and fragments; b: chromatid deletions and gaps; c: chromatid exchanges (quadriradials); d: complicated damages involving chromatid exchanges and deletions.

Dose-effects of Pvu II on the production of chromosomal aberrations

Clastogenic effects of *Pvu* II on AT and normal cells were investigated. Figure 4. 4 shows the frequency of chromosomal aberrations as a function of the concentration of *Pvu* II in SLO (0.06 units/ml) porated AT-PA and N-SW cells harvested 5 or 24 hours (including 3 hours of incubation with 0.04 μg/ml colcemid) after treatment with the enzyme. In contrast to results with Mn, at both sampling times, AT-PA cells exhibited 2 - 4 fold higher frequency of aberrations when compared with normal N-SW cells.

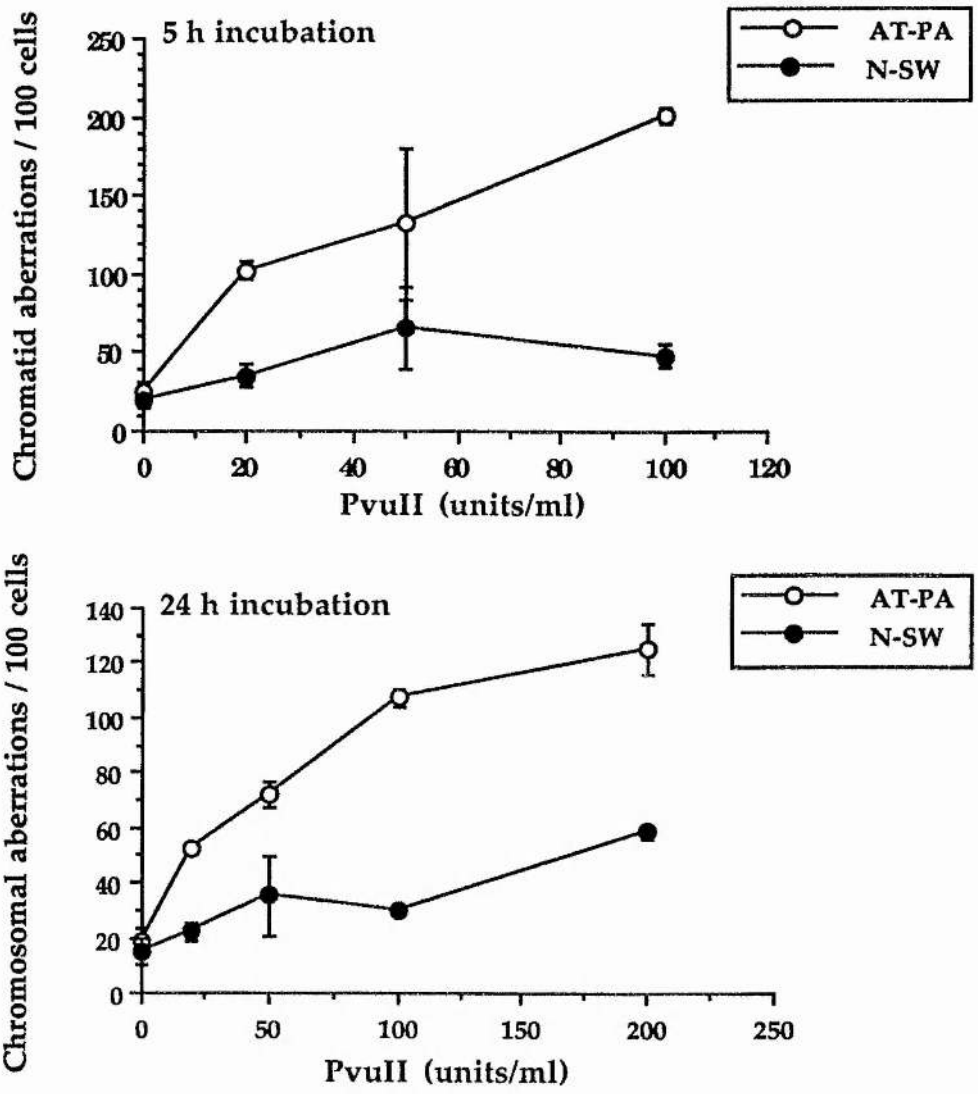

Figure 4. 4. Frequency of chromosomal aberrations in AT-PA and N-SW cells treated with *Pvu* II for 5 (upper panel) and 24 (lower panel) hours. Error bars represent standard error of mean values (see Table 4. 1).

The details of the above results are shown in Table 4. 1. The percentage of the cells of both lines containing aberrations increased proportionally with concentration of *Pvu* II and the frequency of aberrant cells was found to be higher in AT-PA than N-SW cells as illustrated in Figure 4. 5.

Table 4. 1. Metaphase aberrations (CA) per 100 AT-PA and N-SW cells treated with *Pvu* II and SLO.

Cell line	*Pvu* II (units/ml)	Fixation time (h)	No. of cells analysed[a]	% of damaged cells	Chrom[b] exchanges	Chrom[b] deletions	Chromtd[c] exchanges	Chromtd[c] deletions	Chromtd[c] gaps	CA per 100 cells	SEM
AT-PA	0	5	300 (3)	20.3	0	0	0	10.3	14.7	25.0	6.7
	20	5	200 (2)	33.0	0	0	1.5	51.5	49.0	102.0	6.0
	50	5	400 (4)	38.2	0	0	2.7	67.7	62.7	132.2	48.2
	100	5	200 (2)	46.0	0	0	5.0	126.0	66.5	200.5	4.9
N-SW	0	5	300 (2)	16.7	0	0	0	4.3	15.0	19.3	4.0
	20	5	200 (2)	26.0	0	0	0	8.5	26.0	34.5	7.5
	50	5	400 (2)	23.0	0	0	0	33.0	33.0	65.2	25.8
	100	5	200 (2)	27.5	0	0	2.0	15.5	30.5	48.0	7.1
AT-PA	0	24	300 (3)	16.3	0.3	2.3	0.3	4.0	12.0	18.3	4.6
	20	24	200 (2)	25.5	2.5	7.5	7.5	20.0	15.0	52.5	1.5
	50	24	400 (4)	37.7	3.5	7.7	6.5	19.2	32.2	69.1	7.8
	100	24	200 (2)	42.5	6.5	12.0	17.0	45.0	26.5	107.0	3.0
	200	24	200 (2)	47.0	6.0	9.0	16.5	51.0	42.5	125.0	9.0
N-SW	0	24	300 (3)	13.3	0	1.0	0	3.0	11.0	15.0	4.5
	20	24	200 (2)	15.0	3.0	5.0	0	6.0	8.0	22.0	3.0
	50	24	300 (3)	24.3	8.0	7.0	0	4.7	11.3	35.3	14.6
	100	24	200 (2)	21.5	3.5	1.5	0	8.0	17.0	30.0	2.0
	200	24	200 (2)	34.5	17.5	5.5	0	9.0	26.5	58.5	2.5

[a]: Number of experiment in parenthesis; [b]: Chromosome-type; [c]: Chromatid-type

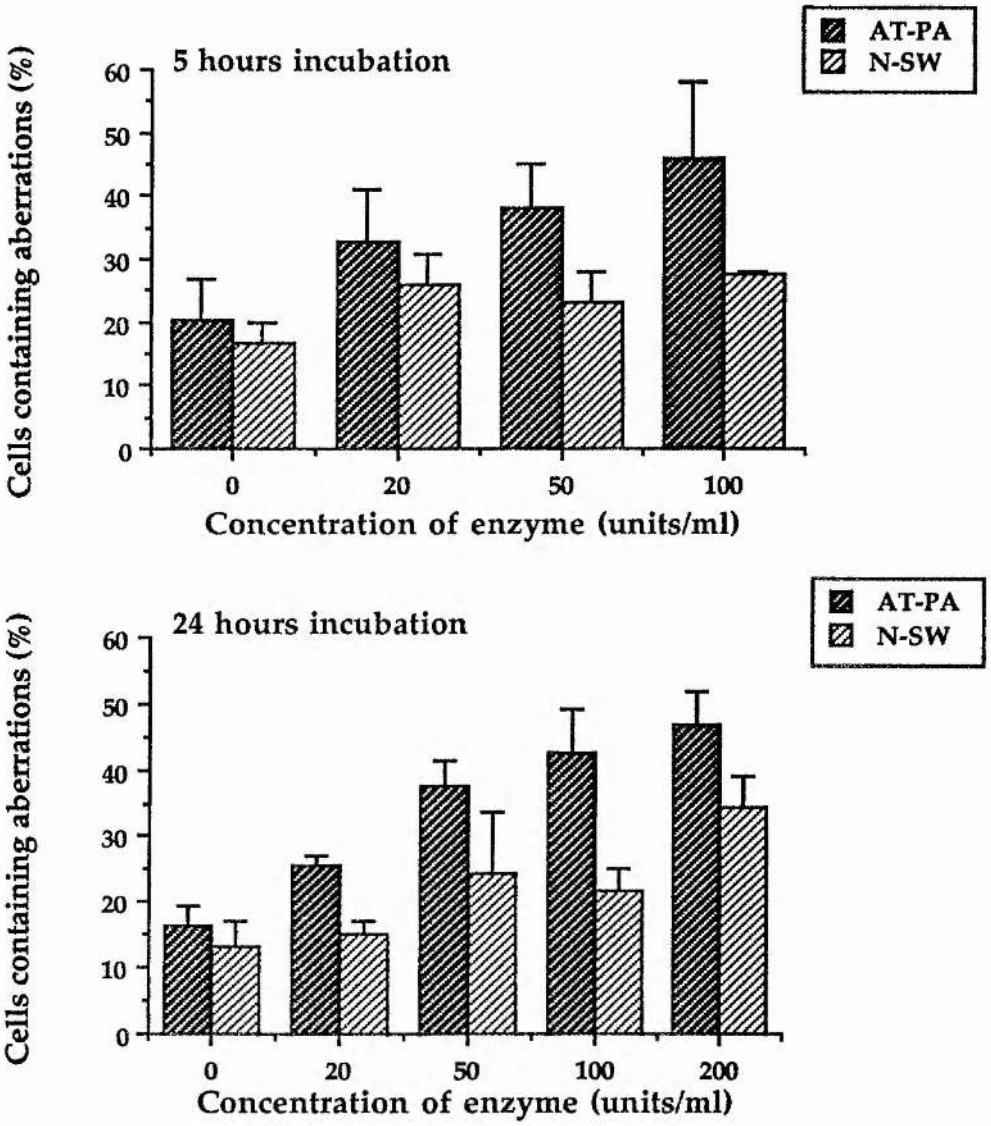

Figure 4. 5. Proportion of AT-PA and N-SW cells containing chromosomal aberrations after 5 (upper panel) and 24 (lower panel) hours after *Pvu* II treatment. Error bars represent standard error of mean values (see Table 4. 1).

At 5 hours after *Pvu* II treatment, the aberrations observed were almost exclusively of the chromatid-type in both AT and normal cell lines. (Table 4. 1). Both chromatid deletions and gaps increased in frequency with increasing concentration of *Pvu* II and appeared at higher frequency in AT-PA than in N-SW cells. Both chromosome- and chromatid-type aberrations were found in *Pvu* II treated AT and normal cells after 24 hours incubation (Table 4. 1). The yield of chromatid-type aberrations after 24 hours

incubation time was dramatically increased in AT-PA cells compared to N-SW cells; however, no significant difference in the chromosome-type aberrations was observed between the two lines (Figure 4. 6).

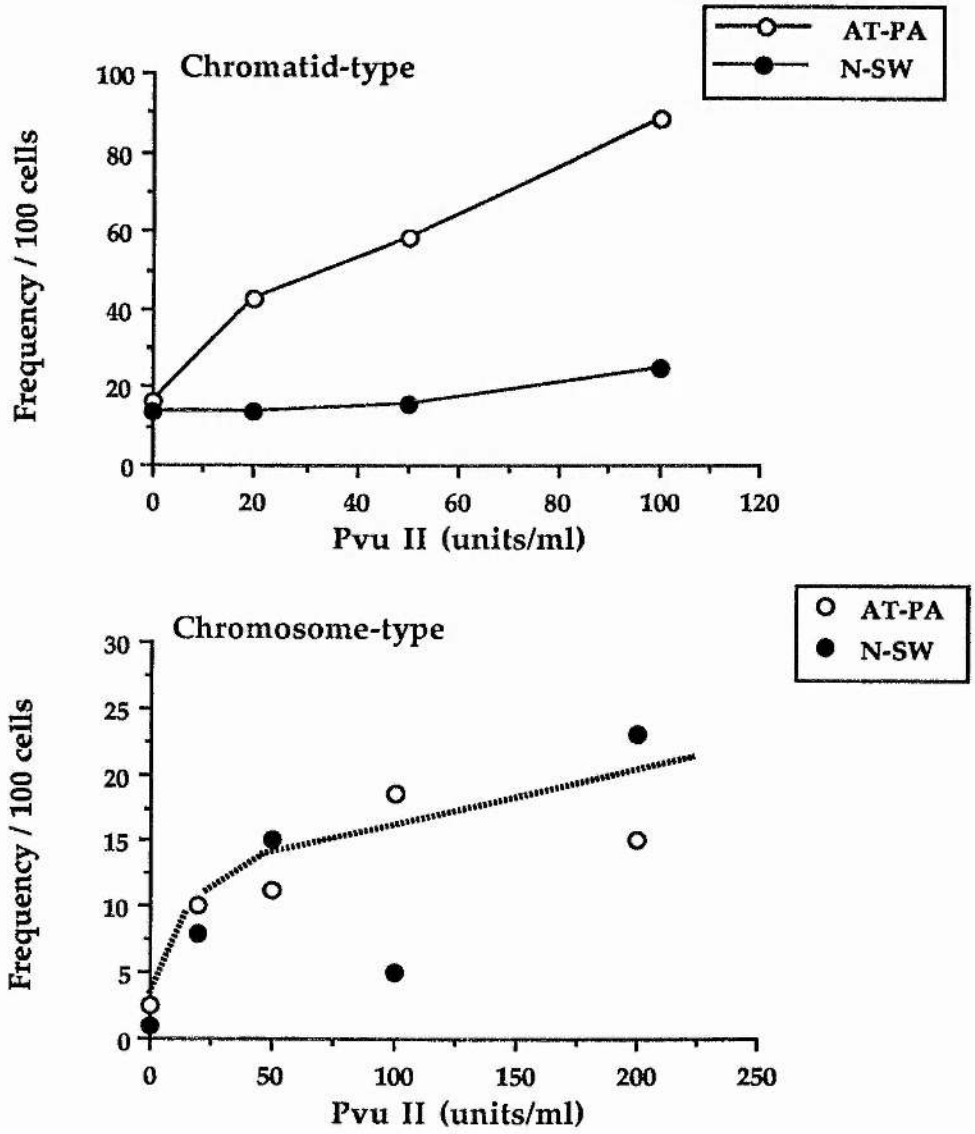

Figure 4. 6. Frequency of chromatid-type (upper panel) and chromosome-type (lower panel) aberrations in AT-PA and N-SW cells treated with *Pvu* II and incubated for 24 hours before fixation. Pooled data from 2 - 4 independent experiments as shown in Table 4. 1.

Comparison of the frequencies of chromosomal aberrations induced by Pvu II in cells harvested at various post-treatment incubated times

The production of *Pvu* II-induced chromosomal aberrations in cells was found to be influenced by post-treatment incubation time. At 50 units/ml of *Pvu* II, the yields of aberrations decreased between 5 to 24 hours time interval, and slightly increased after 30 hours incubation in both AT-PA and N-SW cells (Figure 4. 7). A different time course of chromosomal aberrations was observed between AT and normal cells at 100 units/ml of *Pvu* II (Figure 4. 7). At this concentration of *Pvu* II, AT-PA cells showed a consistent decrease in the frequency of chromosomal aberrations with increasing incubation time, while in N-SW cells the yield of aberrations decreased between 5 and 24 hours interval and increased again at 30 hours, at which time the frequency of chromosomal aberrations caused by *Pvu* II in N-SW cells was found to be higher than that in AT-PA cells (Figure 4. 7). The increased number of aberrations in N-SW cells at 30 hours mainly resulted from an increased number of chromosome-type aberrations, while in AT-PA cells this increase was not apparent (Table 4. 2)

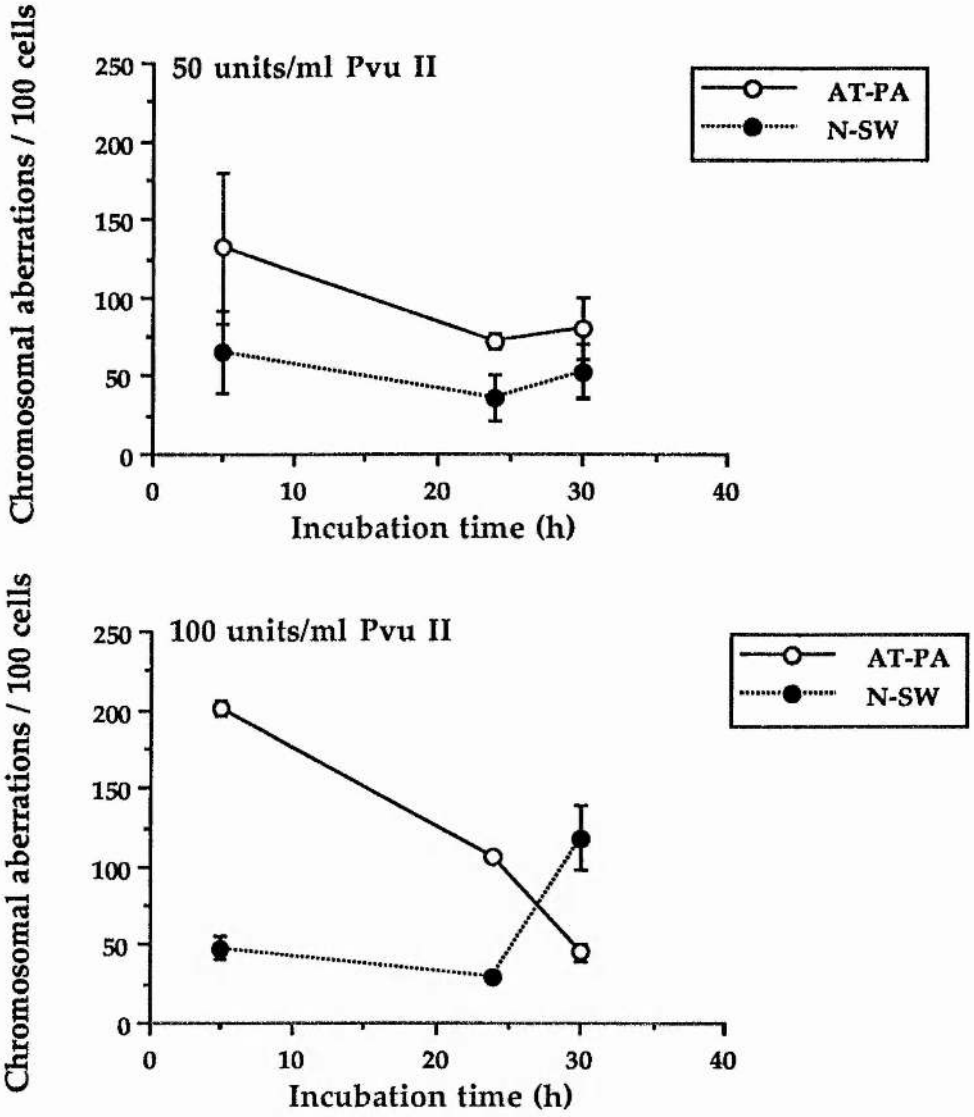

Figure 4. 7. Production of chromosomal aberrations induced by *Pvu* II 50 (upper panel) and 100 units/ml (lower panel) as a function of post-treatment incubation times in AT-PA and N-SW cells. Error bars represent standard errors of mean values (Table 4. 1 and 4. 2).

Table 4. 2. Frequencies of chromosomal aberrations (CA) induced by *Pvu* II in AT-PA and N-SW cells 30 hours after post-treatment incubation. Mean values ± standard errors.

Cell line	*Pvu* II (units/ml)	No. of cells analysed[a]	Chromosome-type aberrations[b]	Chromatid-type aberrations[c]	Chromatid exchanges[d]	Total CA / 100 cells
AT-PA						
	0	300 (3)	2.3 ± 0.9	18.3 ± 2.3	0	20.7 ± 2.1
	20	400 (4)	4.75 ± 5.0	60.5 ± 9.2	2.5 ± 2.6	66.2 ± 6.6
	50	400 (4)	14.2 ± 1.9	59.2 ± 17.9	6.5 ± 3.5	79.8 ± 19.9
	100	300 (3)	8.7 ± 6.0	35.8 ± 5.9	0.67 ± 0.47	45.3 ± 6.0
N-SW	0	400 (4)	0.5 ± 0.5	12.7 ± 3.7	0	13.2 ± 3.5
	20	300 (3)	20.0 ± 20.3	15.5 ± 2.1	0	36.0 ± 19.8
	50	400 (4)	32.7 ± 8.8	20.2 ± 8.6	0.5 ± 0.86	53.0 ± 16.6
	100	300 (3)	90.7 ± 18.7	27.6 ± 18.7	0	118.3 ± 20.8

[a]: Number of independent experiment in parenthesis

[b]: Including chromosome exchanges and deletions

[c]: Including chromatid deletions, gaps and exchanges (shown seperately in column[d])

4. 2. 3. Effects of SLO concentration on induction of chromosomal aberrations by *Pvu* II in AT-PA and N-SW cells

The production of chromosomal aberrations in cells porated with SLO at various concentrations was examined. The results of these experiments are shown in Table 4. 3 and Table 4. 4. for AT-PA and N-SW cells, respectively. The yield of aberrations induced by 50 units/ml of *Pvu* II in AT-PA and N-SW cells increased 2 - 3 times at the higher concentration (0.12 and 0.3 units/ml) of SLO treated cells compared with 0.06 units/ml of SLO, although the percentage of cells containing aberrations was not increased to the same extent, indicating individual cells were porated more efficiently with higher concentrations of SLO, and that the production of chromosomal aberrations corresponds to poration efficiency.

AT cells treated with 100 units/ml of *Pvu* II showed a reduced frequency of aberrations (Table 4. 3), while N-SW cells yielded 4-times more aberrations (Table 4. 4) in response to 0.12 units/ml of SLO poration when compared to those treated with 0.06 unit/ml of SLO. However, the number of metaphases available was dramatically reduced when employing higher concentrations of SLO for either cell lines especially for N-SW line. In AT cells sampled 24 hours after treatment, more chromatid-type aberrations were observed when porated with 0.12 and 0.3 units/ml of SLO compared with 0.06 units/ml of SLO, while chromosome-type aberrations seemed to be not much influenced. In contrast, chromosome-type aberrations were markedly increased in N-SW cells when porated with 0.12 units/ml of SLO compared with 0.06 units/ml of SLO.

Table 4. 3. Effects of SLO concentration on production of chromosomal aberrations (CA) induced by *Pvu* II in AT-PA cells.

Pvu II (units/ml)	Fixation time (h)	SLO (units/ml)	No. of cells analysed[a]	Aberrant cells (%)	Chromosome-type aberration[b]	Chromatid-type aberration[c]	Total CA per 100 cells	SEM
0	5	0.06	300 (3)	20.3	0	25.0	25.0	6.7
0	5	0.3	100 (1)	24	0	30	30	-
50	5	0.06	400 (4)	38.6	0	132.2	132.2	48.2
50	5	0.3	200 (2)	46.0	0.5	380	380.5	64.5
0	24	0.06	300 (3)	16.3	2.6	16.3	18.3	4.6
0	24	0.12	100 (1)	29	3	30	34	-
50	24	0.06	400 (4)	37.7	11.2	57.9	69.1	7.8
50	24	0.12	100 (1)	44	27	68	95	-
50	24	0.3	100 (1)	42	20	82	102	-
100	24	0.06	200 (2)	42.5	18.5	88.5	107.0	3.0
100	24	0.12	100 (1)	46	4	57	61	-

[a]: Number of independent experiment in parenthesis

[b]: Including chromosome exchanges and deletions

[c]: Including chromatid deletions, gaps and exchanges

[d]: Standard errors of means of average total chromosomal aberrations per 100 cells

Table 4. 4. Effects of SLO concentration on production of chromosomal aberrations (CA) induced by *Pvu* II in N-SW cells.

Pvu II (units/ml)	Fixation time (h)	SLO (units/ml)	No. of cells analysed[a]	Aberrant cells (%)	Chromosome-type aberration[b]	Chromatid-type aberration[c]	Total CA per 100 cells	SEM
0	24	0.06	300 (3)	13.3	1.0	14.0	15.0	4.5
0	24	0.12	100 (1)	27	8	31	39	-
50	24	0.06	200 (2)	24.3	15.0	16.0	35.3	14.6
50	24	0.12	22 (1)[e]	50	27.5	40.5	68	-
100	24	0.06	200 (2)	21.5	5	25	30	2.0
100	24	0.12	38 (1)[e]	92	70	60	130	-

[a], [b], [c] and [d] have the same meanings as in Table 4. 3.

[e]: Total number of metaphase scorable

4. 2. 4. Induction of chromosomal aberrations by treatment with *Bam*H I in AT-PA and N-SW cells

Table 4. 5 shows the production of chromosomal aberrations in AT-PA and N-SW cells treated with *Bam*H I. This enzyme was found to induce aberrations in AT-PA cells, although frequencies of aberrations were not as high as those induced by *Pvu* II. Normal cells showed no significant increase over background levels at either 5 or 24 hours. An increase in chromatid-type aberrations rather than chromosome-type aberrations was observed in AT cells. In addition, chromatid exchanges were also found to occur more frequently in AT cells than in normal cells after *Bam*H I treatment (Table 4. 5).

Table 4. 5. Metaphase aberrations (CA) per 100 AT-PA and N-SW cells treated with *Bam*H I and SLO.

Cell line	*Bam*H I (units/ml)	Fixation time (h)	No. of cells analysed[a]	% of damaged cells	Chrom[b] exchanges	Chrom[b] deletions	Chromtd[c] exchanges	Chromtd[c] deletions	Chromtd[c] gaps	CA per 100 cells	SEM
AT-PA	0	5	300 (3)	20.3	0	0	0	10.3	14.7	25.0	6.7
	20	5	200 (2)	16.5	0	0	0	8.0	14.0	22.0	0
	50	5	300 (3)	23.3	0	0	0	16.0	26.0	42.0	6.2
	100	5	200 (2)	16.5	0	0	3.0	25.0	14.5	42.5	18.5
N-SW	0	5	300 (2)	16.7	0	0	0	4.3	15.0	19.3	4.0
	20	5	200 (2)	14.0	0	0	0	3.5	14.0	17.5	8.5
	50	5	400 (4)	17.0	0	0	0	11.5	15.0	26.5	6.7
	100	5	200 (2)	13.0	0	0	0	4.0	13.5	17.5	7.5
AT-PA	0	24	300 (3)	16.3	0.3	2.3	0.3	4.0	12.0	18.3	4.6
	20	24	100 (1)	15.0	1	3	0	1	15	20	-
	50	24	200 (2)	24.5	0	2.5	0	8.5	23.5	34.5	4.5
	100	24	200 (2)	15.0	0	3.5	0.5	7.5	7.0	18.5	3.5
	200	24	125 (1)	32.0	2.4	7.2	7.2	26.4	31.2	74.4	-
N-SW	0	24	300 (3)	13.3	0	1.0	0	3.0	11.0	15.0	4.5
	20	24	100 (1)	12.0	2	3	0	8	6	19	-
	50	24	200 (2)	9.0	0	1	0.5	5.5	4.5	11.5	4.5
	100	24	200 (2)	17.5	2	2	0	5.5	11.0	20.5	7.5
	200	24	125 (1)	19.2	0.8	4.8	0	5.6	11.2	22.4	-

[a]: Number of experiment in parenthesis; [b]: Chromosome-type; [c]: Chromatid-type

4. 2. 5. Comparison of chromosomal sensitivity of AT and normal cells to RE producing dsb with blunt- or cohesive-termini

Table 4. 6. compares the induction of chromosomal aberrations by various restriction enzymes, either causing blunt-ended (*Pvu* II and *Eco*R V) or cohesive-ended (*Bam*H I and *Pst* I) dsb, in AT-PA, AT-KM and N-SW cell lines. Cells were porated with 0.06 units/ml of SLO in the presence of 50 units/ml of RE and harvested after a 5 hour post-treatment incubation (4 hours incubation with colcemid). The proportion of aberrant cells, for all the cell lines, did not significantly differ between different enzyme treatments, although the production of aberrations was markedly different when different enzymes were compared. *Pvu* II was found to be most effective in the induction of chromosomal aberrations in both AT and normal cells. The blunt-ended dsb produced by *Pvu* II and *Eco*R V caused a higher frequency of aberrations than those produced by *Bam*H I and *Pst* I. It was noted that higher frequencies of aberrations were induced by *Bam*H I and *Pst* I in the two AT cell lines than in the normal cell line (N-SW).

Table 4. 6. Freqencies of chromatid aberrations induced by treatment with 50 units/ml restriction endonucleases in AT-KM, AT-PA and N-SW cells 5 hours after post-treatment incubation. Mean values ± standard errors.

Cell line	RE (50 units/ml)	No. of cells analysed[a]	Aberrant cells (%)	Chromatid aberrations per 100 cells
AT-KM				
	0	200 (2)	20.5 ± 1.5	23.5 ± 1.5
	Pvu II	300 (3)	38.0 ± 5.3	196.3 ± 43.7
	Pst I	200 (2)	36.0 ± 1.0	72.5 ± 8.5
	*Bam*H I	300 (3)	27.6 ± 10.9	55.3 ± 16.7
AT-PA				
	0	300 (3)	18.0 ± 0	22.7 ± 0.5
	Pvu II	400 (4)	44.2 ± 8.8	123.5 ± 45.9
	*Eco*R V	200 (2)	40.5 ± 2.5	75.5 ± 4.5
	Pst I	200 (2)	36.9 ± 12.4	47.0 ± 20.0
	*Bam*H I	300 (3)	43.3 ± 8.7	65.0 ± 12.0
N-SW				
	0	400 (4)	12.2 ± 5.5	13.0 ± 5.9
	Pvu II	300 (3)	22.3 ± 1.7	54.0 ± 11.1
	*Eco*R V	100 (1)	12	23
	Pst I	200 (2)	17.5 ± 3.5	22.5 ± 2.5
	*Bam*H I	300 (3)	23.0 ± 4.6	34.7 ± 3.7

[a]: Number of independent experiment in parenthesis

4. 2. 6. Production of dsb in the DNA of cells by RE treatment

Dsb induced by 500 units/ml of *Pvu* II and *Bam*H I in AT-PA and N-SW cells porated by 0.06 units/ml SLO were quantified using neutral filter elution. Results are shown in Figure 4. 8. *Pvu* II caused a level of DNA elution which corresponded to approximately a 5 - 6 fold higher frequency of dsb in N-SW than in AT-PA cells. *Bam*H I showed less effectiveness in the induction of dsb than *Pvu* II in N-SW cells, but appeared to be as effective as

Pvu II in AT-PA cells. The production of dsb in *Pvu* II (500 units/ml) treated AT-PA and N-SW cells can be estimated from a γ-ray dose-effect curve (Figure 3. 7) to be equivalent to the dsb induced by about 2 and 10 Gy γ-rays, i.e., about 80 and 400 dsb/cell, respectively (Blöcher and Pohlit 1982).

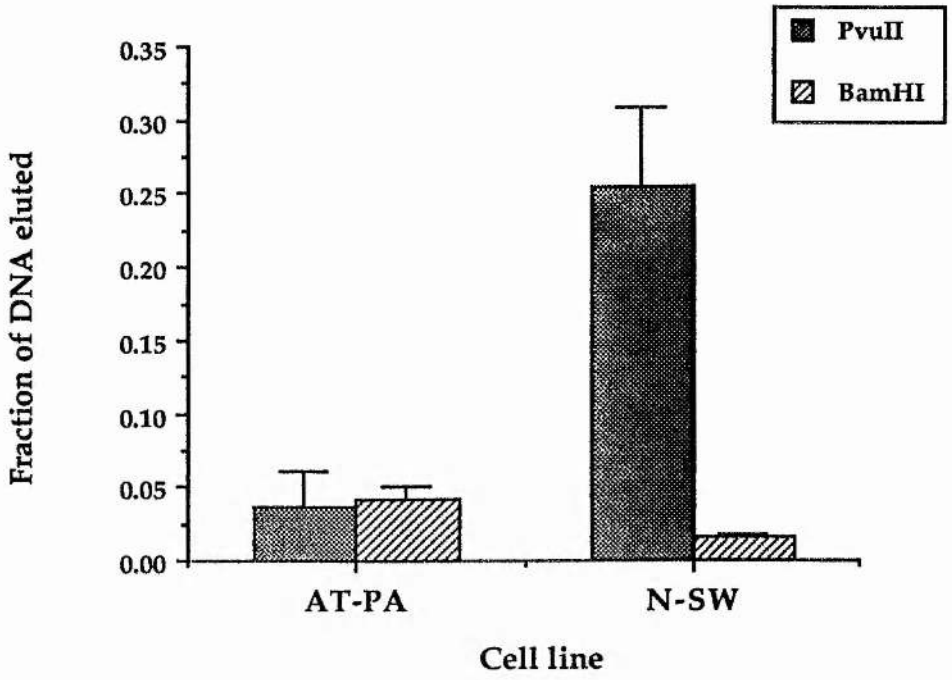

Figure 4. 8. Double strand breaks induced by *Pvu* II and *Bam*H I (both at 500 units/ml) in SLO (0.06 units/ml) porated AT-PA and N-SW cells after 4.5 hours post-treatment incubation. Data are pooled from 4 (*Pvu* II) and 2 (*Bam*H I) independent experiments. Vertical bars represent standard errors of mean values.

To test whether the higher production of dsb in N-SW in comparison to AT-PA cells is the result of a different uptake of RE by the cell lines, AT cells were porated with a 5 times higher concentration of SLO (0.3 units/ml) than that used for N-SW cells (0.06 units/ml), and the frequency of dsb examined after 4.5 hours incubation. It was observed that normal cells appeared to be lysed at this concentration of SLO (0.3 units/ml), while AT-PA cells were less affected. Figure 4. 9 shows the results obtained with

various concentrations of *Pvu* II. Compared with lower SLO concentration (0.06 units/ml), 0.3 units/ml SLO treatment resulted in an increase in the yield of dsb in AT-PA cells, the level of which was similar to that observed in N-SW porated with 0.06 units/ml SLO. The induction of dsb initially increased with increasing concentration of *Pvu* II followed by a saturation at higher enzyme concentrations.

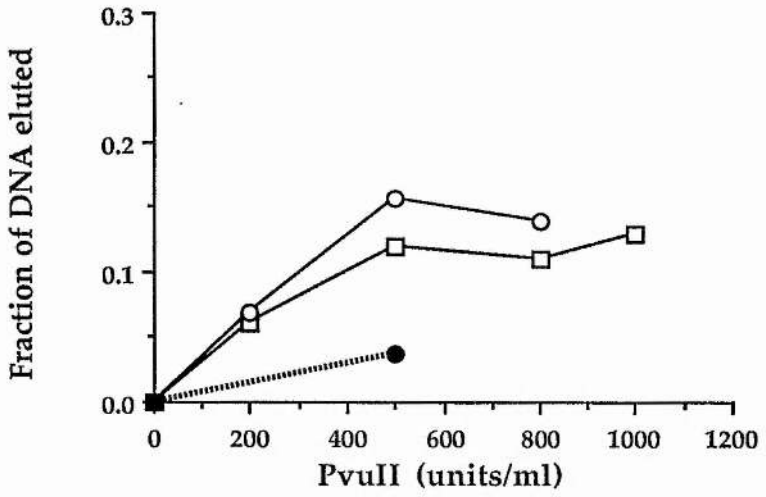

Figure 4. 9. Induction of dsb by various concentrations of *Pvu* II in AT-PA cells (Circles), porated with SLO at either 0.06 (dotted line) or 0.3 (solid line) units/ml, and N-SW (squares) porated with 0.06 units/ml of SLO. Data were pooled from at least 3 independent experiments.

4. 2. 7. Cell poration assay

In an attempt to investigate the efficiency of SLO in cell membrane poration, the leakage of ^{3}H-methionine labelled cellular proteins was examined. Cells were exposed to various concentrations of SLO for 5 min, or to 0.06 units/ml of SLO for various times (1 - 30 min), in a total volume of 0.5 ml. The cells were then centrifuged and the supernatants (about 0.5 ml) were determined for radioactivity with 4 ml of Filter-Count^{TM}. The

supernatant was also ultrafiltered using an Amicon 10 filter by centrifugation at 8,000 rpm (7,700 x g) for 1.5 hours, and ^{3}H-activity in the filtrate and on the filter were then counted.

More than 80% of radioactivity was retained by Amicon 10 filter in both AT-PA and N-SW cell lysates (Figure 4. 10), indicating that the majority of labelled molecules were proteins of MW > 10 Kdal. Figure 4. 11 shows an example of a time-course experiment for exposure to SLO. The release of ^{3}H-labelled proteins with time was found to be 4 - 5 fold higher in N-SW cells than AT-PA cells following poration with a single concentration of SLO (Figure 4. 11).

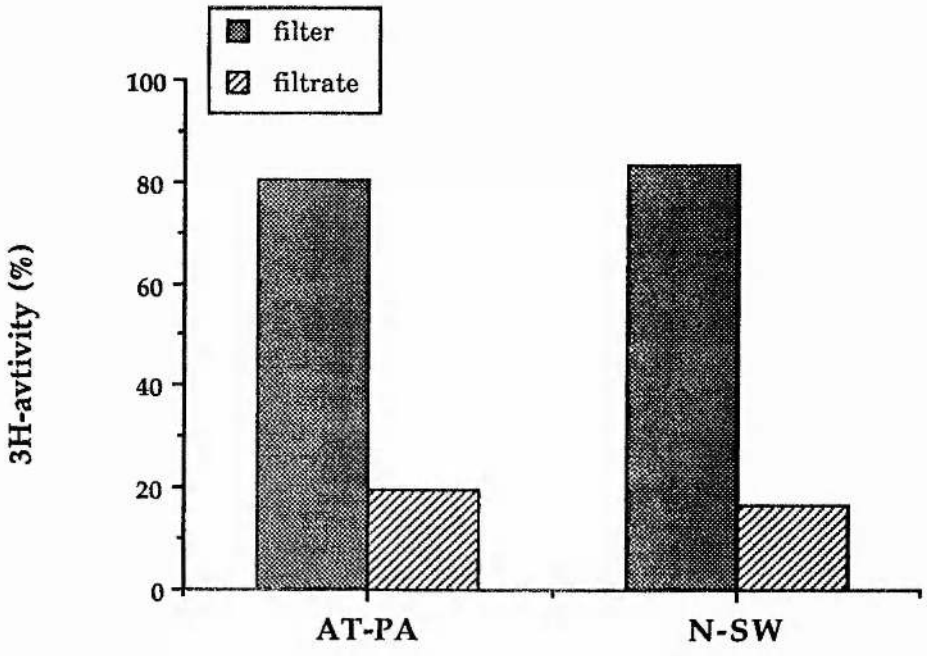

Figure 4. 10. Percentage of incorporated radioactivity retained by the filter (MW > 10 Kdal) or in the filtrate (MW < 10 Kdal). AT-PA or N-SW cells were exposed to 0.06 units/ml of SLO for 5 min.

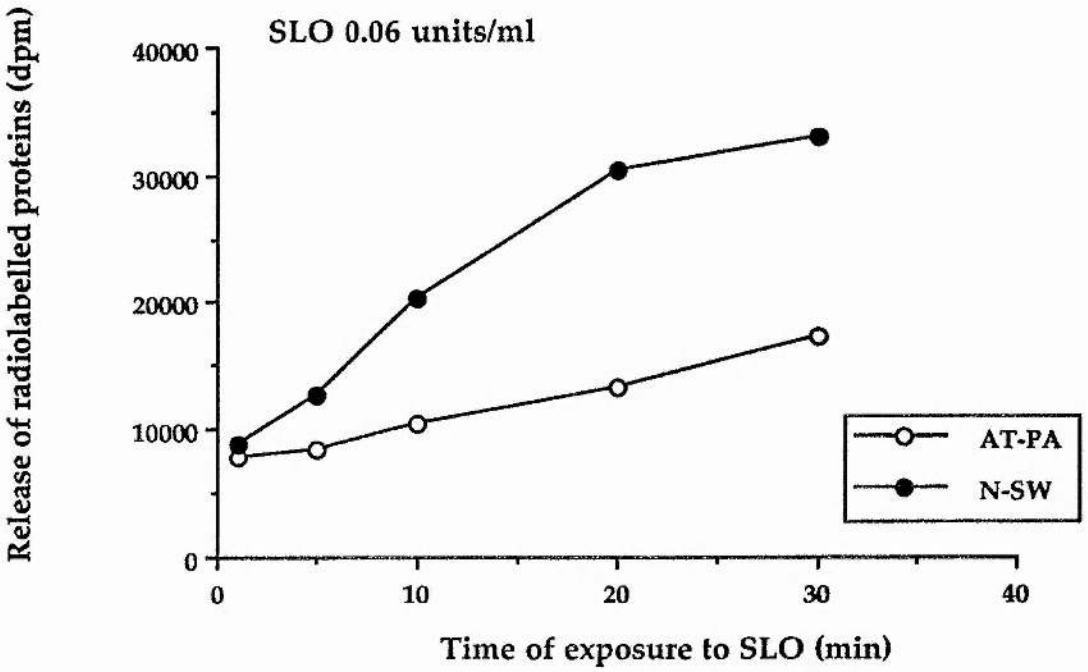

Figure 4. 11. Release of ^{3}H-methionine labelled cellular proteins from porated AT-PA and N-SW cells as a function of exposure time to SLO.

Figure 4. 12 shows that the releases of radiolabelled proteins from AT-PA, AT-KM and N-SW cells are dependent on the concentration of SLO. Both AT cell lines showed a lower rate of cellular protein leakage than N-SW cells. These results indicate that N-SW cells are porated by SLO more efficiently than AT-KM and AT-PA cells.

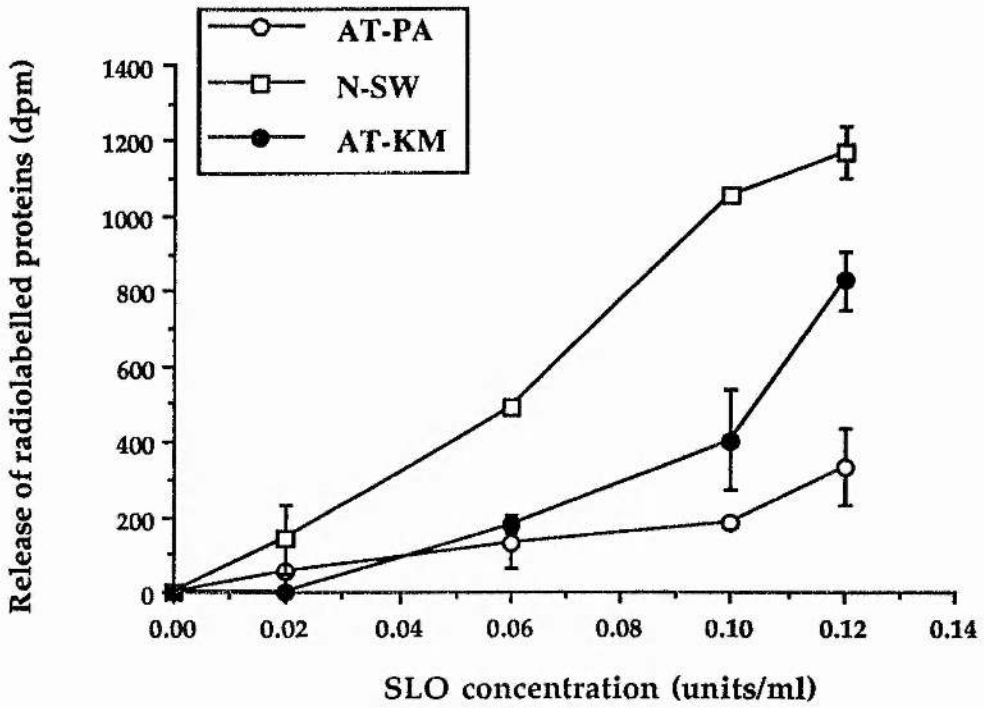

Figure 4. 12. Release of ^{3}H-methionine labelled cellular proteins from porated AT-KM, AT-PA and N-SW cells as a function of SLO concentration. The cells were exposed to SLO for 5 min in a total volume of 0.5 ml. Data pooled from 2 to 4 independent experiments. Vertical bars represent standard errors of mean values.

4. 2. 8. Assay of stability and activity of RE *in vitro*

The activity of RE after purification was investigated by determining the ability of RE to digest plasmid pBR322 in simulated cellular ionic conditions (in boiled cell extracts) *in vitro* (see Chapter 2 section 2. 3). Storage of diluted RE (1 unit/µl) in a solution of HBSS/BSA at 4 °C overnight after purification did not reduce the activity of *Pvu* II, while slightly decreased the activity of *Bam*H I (data not shown). Figures 4. 13 and 4. 14 show the results obtained for *Pvu* II and *Bam*H I, respectively, after incubation at 37 °C with the extracts (before addition of plasmid) for various time. After purification, the minimum amount of *Pvu* II (Figure 4. 13) and *Bam*H I (Figure 4. 14) required to completely linearize pBR322 at 37 °C for 1

hour were found to be 0.125 and 0.5 units, respectively, before the incubation and these remained unchanged after incubation for up to 19 hours for both of the enzymes. After a 24-hour incubation, the activities of *Pvu* II and *Bam*H I were similarly slightly reduced. These results provide evidence that *Pvu* II and *Bam*H I are both relatively stable under non-optimal conditions.

Using the same methods described above, the activity after RE purification and stability at 37 °C of *Pst* I and *Eco*R I were examined. The activities after purification for these enzymes were observed to be similar each other, which were slightly lower than that of *Bam*H I and much lower than that of *Pvu* II. The stability were found in the following order *Pvu* II = *Bam*H I > *Pst* I > *Eco*R I.

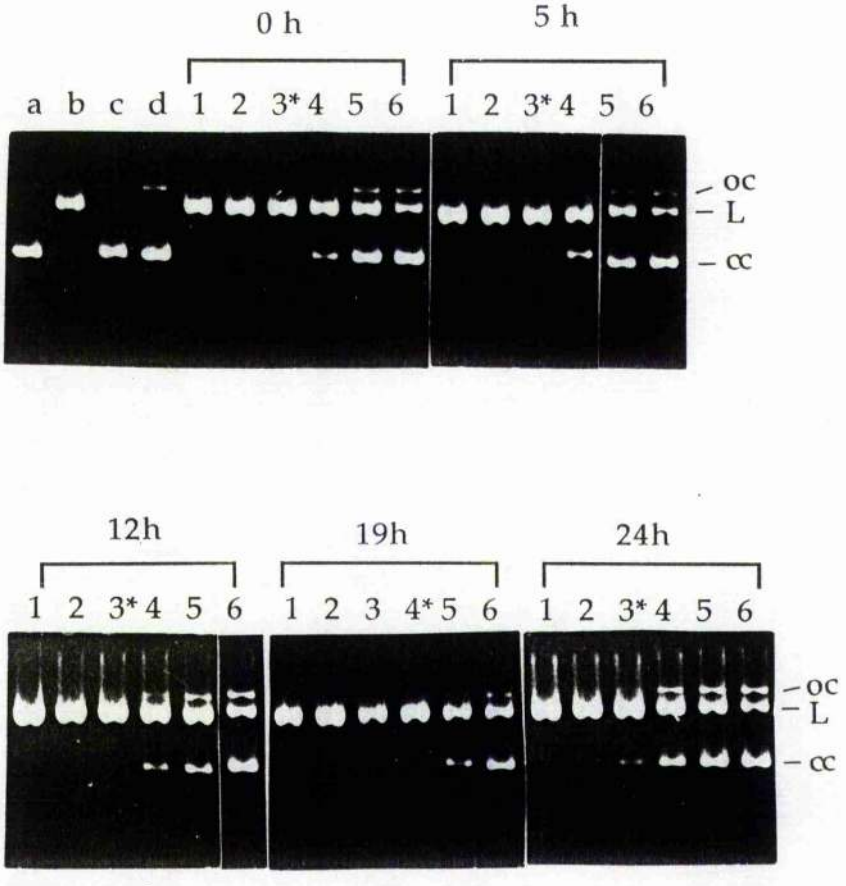

Figure 4. 13. Agarose gel assays of the stability of *Pvu* II following purification and incubation at 37 °C with the cell extracts for various time before addition of pBR322. Reactions (after addition of pBR322) were carried out at 37 °C for 1 hour. Lane a: pBR322; lane b: pBR322/*Pvu* II; lane c: pBR322 incubated with the extracts for 24 hours; lane d: pBR322 incubated with HBSS/BSA for 24 hours. Lanes 1 to 6: *Pvu* II 0.5, 0.25, 0.125, 0.06, 0.03 and 0.015 units, respectively. cc: closed circles; L: linear; oc: open circles. * RE amount for complete digestion of pBR322.

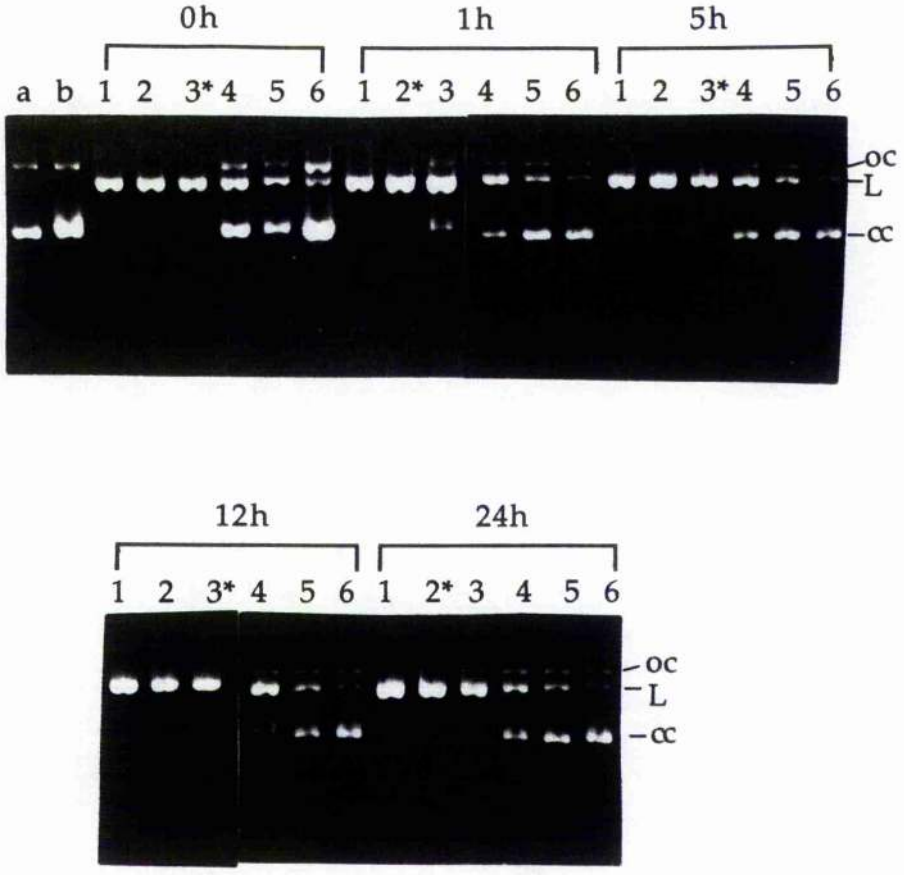

Figure 4. 14. Agarose gel assays of the stability of *Bam*H I after purification and incubation at 37 °C with cell extracts for various time before addition of pBR322. Reactions (after addition of pBR322) were carried out at 37 °C for 1 hour. Lane a: pBR322; lane b: pBR322 incubated with the extracts for 24 hours. Lanes 1 to 6: *Bam*H I 2, 1, 0.5, 0.25, 0.125 and 0.06 units, respectively. cc: closed circles; L: linear; oc: open circles. * RE amount for complete digestion of pBR322.

4. 3. Discussion

Chromosomal sensitivity of AT and normal cells to RE

Treatment of human lymphoblastoid cells with RE, with the aid of cell poration by SLO, induced all types of chromosomal aberrations (both exchanges and breaks) similar to those seen at metaphase after ionizing radiation (Figure 4. 3). The production of chromosomal aberrations by RE mimics the cytogenetic effects of radiation in that chromatid aberrations are induced in G_2 cells (assumed from the 5 h fixation samples) and both chromosome- and chromatid-type aberrations in G_1 cells (assumed from samples fixed at 24 h or later time). AT cells (AT-PA) exhibited a higher frequency of chromosomal aberrations when compared with normal (N-SW) cells, at either 5 or 24 hours fixation times (Figure 4. 4). The production of chromosomal aberrations showed a RE dose-dependent relationship in both AT-PA and N-SW cell lines. Correspondingly, the number of cells containing chromosomal damages increased with increasing concentrations of RE in both AT-PA and N-SW cells, and the frequency was again higher in AT-PA cells (Figure 4. 5). These results suggest that AT cells possess a defect in processing of dsb that convert higher number of dsb into chromosomal aberrations.

Despite the increased induction of chromosomal aberrations in AT-PA cells, the induction of dsb by *Pvu* II was found to be approximately 5-fold lower in AT than in normal cells when porated with SLO at a given concentration (Figure 4. 8). Results of efforts to assay cell membrane poration showed a 5 - 6 fold lower poration efficiency by SLO in both AT-PA and AT-KM cells compared with normal cells (Figures 4. 11 and 4. 12). On the other hand, the production of dsb could be increased by increasing SLO concentration in AT cells (Figure 4. 9). Consistent with the elevated production of dsb, an increased frequency of chromosomal aberrations was

found in both AT and normal cell lines when SLO concentration was increased. These findings support the suggestion that lower yields of dsb may be a result of a less efficient poration by SLO in AT cells. Nevertheless, AT-PA cells still showed a 2 - 4 fold higher frequency of chromosomal aberrations induced by *Pvu* II compared with normal (N-SW) cells when porated at 0.06 units/ml SLO (Figure 4. 4 and Table 4. 1). The reason for the reduced poration efficiency in the AT cell lines is not understood. In fact, a similar high poration efficiency by SLO over that of AT cells has been determined in another normal human lymphoblastoid cell line (N-DW) (data not shown). This may be an indication that AT cells are generally less sensitive to SLO than normal cells. Higher concentrations of SLO, however, could not be used for the cytogenetic experiments since the mitotic index of cells, particularly for normal cells, treated at SLO concentration in excess of 0.06 units/ml fell to an unacceptable level. These observations introduce some uncertainty into the conclusions, but suggest that the frequency of aberrations for a given concentration of RE in AT cells might have been even higher than in N-SW had the poration been exactly equivalent in the cell lines.

In contrast to metaphase chromosomal aberrations, the micronucleus (Mn) assay gave conflicting results with respect to the sensitivity of AT and normal cells to *Pvu* II. The background frequency of Mn in cells treated with SLO alone was remarkably high in AT-PA cells (Figure 4. 1). Although *Pvu* II treatment apparently gave rise to higher levels of Mn when compared with *Eco*R I in either AT or normal cells, the increases of Mn with increasing RE concentrations were small (Figure 4. 2), showing that the Mn assay is a less sensitive assay than chromosomal aberration analysis. As previously discussed, the yield of RE-induced dsb is lower in AT cells than in normal cells. 500 units/ml of *Pvu* II yielded dsb equivalent to those induced by about 2 and 10 Gy γ-ray in AT and normal cells, respectively. In

addition, the high background Mn in AT cells may conceal some chromatin fragments which would have resulted in individual micronuclei had the background been low. Therefore, the Mn assay seems not suitable for cells which show a high background Mn, especially when the production of DNA breaks is low. The reason for the high background found in AT cells after SLO poration is not clear. A strong inhibition of DNA replication caused by SLO has been observed, which points out the cytotoxicity of SLO on cells (see Chapter 6).

As discussed above, only chromatid type aberrations were found at 5 hours after RE treatment in AT and normal cell lines, indicating that aberrations arise from dsb generated in G_2 phase cells. It is noteworthy that the total number of aberrations induced by *Pvu* II in both AT-PA and N-SW cell lines at 5-hour sampling time was markedly higher than that at the 24-hour sampling time (Table 4. 1), as was observed in γ-irradiated cells (Table 3. 1). Similar results have also been found in wild-type V79 and radiosensitive *irs*-2 mutant cells: a 4-hour post-treatment incubation resulted in an increased level of chromosomal aberrations over that obtained from an 18-hour post-treatment incubation (Bryant et al 1993). The difference was more profound in AT-PA cells although the levels of aberrations were still higher in AT than normal cells after a long period incubation (30 hours). At a high concentration of *Pvu* II (100 units/ml), AT cells harvested at 30 hours yielded a decreased frequency of chromosomal aberrations, while the frequency obviously increased in N-SW cells (Figure 4. 7). The increased number of aberrations in the normal cells were found to consist mainly of an increased number of chromosome-type aberrations (Table 4. 2), indicating that these chromosomal damages were formed from DNA lesions caused at G_1 phase by RE.

A cell cycle block resulting from DNA lesions may delay the expression of chromosomal aberrations in metaphase. To ascertain this

possibility, the effect of SLO poration and RE treatment on cell cycle progression was monitored by a flow cytometric technique. A small increase of population of G_2 phase cells was found in N-SW cells but not in AT-PA cells 24 hours after treatment with SLO (0.06 units/ml) and *Pvu* II (100 units/ml) (Figure 6. 8). The G_2 delay caused by SLO plus *Pvu* II, however, was not significantly different from that caused by SLO alone (Figure 6. 8). A plausible interpretation for the decreased frequency of aberrations in AT cells after 30 hours incubation is that, like in the case of ionizing radiation, AT cells may be less able to tolerate heavier dsb damage. Therefore, many damaged AT cells may be not able to progress through cell cycle.

The fact that chromatid aberrations are induced by treatment with RE (*Pvu* II and *Bam*H I) in G_1 phase (harvested at 24 hours) possibly indicates an intracellular persistence of the enzyme activity into the S-phase. Costa and Bryant (1990a) showed by measuring the production of dsb that *Pvu* II acts inside cells for a relatively long period of time in hamster cells. The stability of *Pvu* II was also determined in this study and a slight reduction in the activity of *Pvu* II in digesting plasmid DNA after 24 hours incubation in an *in vitro* system was observed. However, a striking result was that at the 24 hours sampling time, AT-PA cells showed high levels of chromatid aberrations compared with N-SW cells, whereas the frequencies of chromosome-type aberrations were not significantly different in the two lines. These results correlate well with those in AT and normal cells exposed to γ-rays (Table 3. 1). The level of chromatid exchanges was found to increase with an increasing concentration of *Pvu* II at either 5 or 24 hours sampling times, while in N-SW cells, chromatid exchanges only occurred at a higher concentration of *Pvu* II and at the 5 h fixation time. In addition, AT cells were found to exhibit multiple chromosomal aberrations which often consisted of many exchanges. The presence of a small number of chromatid

exchanges may indicate a defective restitution of chromosomes in AT cells which may result from misrepair of DNA lesions (Taylor 1978).

Clastogenicity of RE producing dsb with either blunt- or cohesive termini

The results in Table 4. 2 show that both normal and AT cells exhibit lower frequencies of chromosomal aberrations when treated with *Bam*H I compared to *Pvu* II, either at 5 or 24 hours sampling times. This observation agrees with the previous reports for CHO cells (Bryant 1984, Natarajan and Obe 1984, Bryant and Christie 1989). *Bam*H I also showed a marked reduction in dsb detected in N-SW cells when compared with *Pvu* II and this is consistent with the observations of lower level of accumulation of dsb in Chinese hamster cells by *Bam*H I than that by *Pvu* II (Costa and Bryant 1991a). This effect has been interpreted as a result of rapid rejoining of dsb (as two single strand breaks) induced by *Bam*H I since the staggered ends may facilitate the linking up of broken strand of DNA (Costa and Bryant 1991a). Therefore, the lower level of chromosomal aberrations induced by *Bam*H I compared with *Pvu* II in normal cells is explained by the smaller number of *Bam*H I-induced dsb available to be converted into chromosomal aberrations.

In contrast, the production of dsb induced by *Bam*H I and *Pvu* II in AT-PA cells was found not much different (Figure 4. 8), although the frequency of chromosomal aberrations (at 5 hours fixation time) induced by *Pvu* II were found to be 3 - 4 times higher than those induced by *Bam*H I. This implies that another mechanism may exist, which leads to a lower frequency of conversion of cohesive-ended dsb into chromosomal aberrations, and which may involve different repair pathways for blunt- and cohesive-ended dsb.

Another restriction enzyme causing cohesive-ended dsb, *Pst* I, when compared with RE causing blunt-ended dsb (*Pvu* II and *Eco*R V), results in

lower frequency of chromosomal aberrations. *Eco*R V caused less aberrations when compared with *Pvu* II, although *Eco*R V possess a higher theoretical cutting frequency than *Pvu* II. In addition, when *Pst* I and *Bam*H I are compared, it appeared that the frequency of cutting sites is not an absolute determining factor for the clastogenicity of RE, since the cutting sites of *Bam*H I have been estimated to be more frequent than those of *Pst* I, but *Bam*H I did not always give rise to higher frequency of aberrations when compared to *Pst* I (Table 4. 6).

The lower yield of chromosomal aberrations caused by RE producing cohesive-ended dsb may be attributable to two factors. Firstly, the induction of dsb in DNA by these enzymes, i.e., the activity and stability of RE under cellular conditions. Secondly, the processing or repair of dsb.

Plasmid assay of RE activity following purification was carried out in boiled whole cell extracts, used to simulate the ion strength conditions inside the cells. The results showed that the minimum concentration of RE required to digest pBR322 completely was higher for *Bam*H I than for *Pvu* II, i.e., the activity of *Bam*H I detected in the conditions was lower than that of *Pvu* II by a factor of 4 (Figure 4. 13 and 4. 14). However, both the enzymes seemed to be similarly stable in the non-optimal storage conditions used. When taking account of the lower activity of *Bam*H I in a comparative experiment, a 4-fold higher concentration of *Bam*H I than of *Pvu* II was used. Even under these conditions, *Bam*H I failed to induce a comparable frequency of chromosomal aberrations to that induced by *Pvu* II (data not shown). On the other hand, *Eco*R V produced a higher frequency of aberrations than *Pst* I and *Bam*H I in all cell lines, although the activity of *Eco*R V was found to be even less than that of *Bam*H I. Therefore, the different activities of RE in cutting chromatin inside cells may partly, but not absolutely, account for the different clastogenicity of RE. Evidence of rejoining of dsb in plasmids by human cell extracts suggested that breaks

with cohesive ends are rejoined more efficiently than blunt-ended breaks (Ganesh et al 1993). This finding supports the suggestion that cohesive-ended dsb are repaired more rapidly than blunt-ended dsb (Bryant 1984, Costa and Bryant 1989). The lower clastogenic effect of cohesive-ended dsb may therefore also be a result of a more efficient processing of this type of dsb in comparison to blunt-ended dsb.

Nevertheless, both AT cell lines (AT-PA and AT-KM) exhibit a higher frequency of aberrations arising from cohesive-ended dsb induced by either *Bam*H I or *Pst* I when compared with normal cells. This result indicates that AT cells are also defective in processing dsb with cohesive termini, as found in the case of *Kpn* I sites in pSV2*gpt* (Cox et al 1984) and other cohesive-ended dsb in plasmid DNA (North et al 1990). This has been suggested to be due to an increased risk of strand exposure of dsb in AT cells than in normal cells. Thus in essence, AT cells may convert a higher number of cohesive-ended dsb into blunt-ended dsb, thereby causing an enhanced level of chromosomal aberrations. Such conversion may involve either an enhanced activity of an exonuclease (Cox et al 1984) or a lack of normal protection of the strand end by a functional DNA binding protein(s) in AT cells (North et al 1990). The implication of the present results is that cohesive-ended dsb may be processed differently in AT cells from that in normal cells. This raises the question as to whether the "clean" ends, either blunt or staggered, are subjected to end-modification prior to ligation, instead of a direct rejoining by a ligase. An attempt to address this question has been made by the use of a DNA repair inhibitor ara A and the results are presented in Chapter 5.

Comparison of AT with other radiosensitive mutant cells in response of RE-induced dsb

Most of the rodent mutant cell lines investigated for their sensitivity to RE-induced dsb showed an enhanced chromosomal or cellular sensitivity to RE when compared with their parental lines (Bryant et al 1987, 1993, Darroudi and Natarajan 1989, Cortés and Ortiz 1991, Giaccia et al 1990). Unlike *xrs*-5 cells (Costa and Bryant 1991b), *irs*-2 cells accumulated dsb induced by *Pvu* II to the same extent as V79 cells during the incubation interval (up to 3 hours) tested (Bryant et al 1992), indicating a similar degree of cutting and rejoining of dsb occurring in *irs*-2 cells. Nevertheless, a higher than normal frequency of chromosomal aberrations is exhibited in *irs*-2 cells after *Pvu* II treatment (Bryant et al 1992).

In this study, AT cells accumulated even less dsb than normal cells 4.5 hours after *Pvu* II treatment, probably due to a reduced amount of RE introduced by the lower efficiency of SLO poration, while yielding 2 - 5 times higher frequency of chromosomal aberrations than normal cells. These findings indicate, as in the case of ionizing radiation, that the apparent frequency of dsb does not correspond to the number of chromosomal aberrations subsequently detected.

A common point with respect to the sensitivity to RE observed in the radiosensitive mutant cells is that they show an increased sensitivity to dsb with staggered ends than normal phenotype cell lines, in addition to showing hypersensitivity to blunt-ended dsb. For example, *xrs*-5 cells have been found to produce more chromosomal aberrations than CHO K1 cells when treated with *Bam*H I and *Eco*R I, while showing 2 - 3 times more aberrations when treated with *Pvu* II and *Eco*R V (Bryant et al 1987). *Scid* cells in a clonogenic assay were approximately 3-fold more sensitive to the blunt-ended dsb produced by *Rsa* I and 3 - 4 fold more sensitive to cohesive-ended dsb produced by *Sau*3A I, when compared to wild type CB-17 mouse

cells (Chang et al 1993). Similarly, XR-1 cells were found in a clonal assay to be more sensitive to *Sau*3A I, as well as to *Alu* I (producing blunt ends), than its parental CHO line (Giaccia et al 1990).

The results for AT-PA and AT-KM cells studied here show that both AT cell lines are more sensitive to cohesive-ended dsb generated by *Bam*H I and *Pst* I in the production of chromosomal aberrations, when compared with normal N-SW cells. The fact that radiosensitive cell lines are hypersensitive to both blunt- and cohesive-ended dsb may suggest an abnormal processing of all type of dsb in these cells.

Formation of chromosomal aberrations may occur as a result of incorrectly repaired dsb, a defect which has been demonstrated in AT cells (Cox et al 1984). The fidelity of dsb repair, however, has been shown to be normal in *irs*-2 cells as tested by the rejoining of *Kpn* I cut dsb in a selectable gene (*gpt*) in transfected plasmids (Debenham et al 1988). In this respect, *irs*-2 cells are different from AT cells in that AT cells have been shown to be deficient in rejoining dsb by using the same assay system (Cox et al 1984, Debenham et al 1985). In fact, although both *irs*-2 and AT cells produced higher levels of chromosomal aberrations than the normal control cells, the appearance of types of aberrations seem to be different. Following G_1 treatment with RE, *irs*-2 cells showed an increase in all types of aberrations, both chromosome- and chromatid-type, a phenomenon which is consistent with that observed following G_1 irradiation of this cell line. In AT cells, the increased chromosomal sensitivity to RE of G_1 cells was predominantly in the form of chromatid aberrations and this is in agreement with the observations of AT cells following G_1 irradiation (Table 3. 1).

Taken together, the hypersensitivity of radiosensitive mutant cell lines to RE-induced dsb may involve several different mechanisms which involve the ability to rejoin dsb (e.g., for *xrs*, XR-1, *scid* cells), the fidelity of

rejoining of dsb (for AT cells) and other mechanisms which as yet remain unclear (for *irs*-2 cells).

To summarise, the data show that AT cells are characterised by a DNA dsb processing defect which converts a higher number of blunt- or cohesive-ended dsb into chromosomal aberrations than is the case for the normal N-SW cell line. The results also support the notion that AT cells are hypersensitive to ionizing radiation as a result of a dsb processing defect.

Chapter V

Effects of Ara A on the Clastogenicity of Restriction Endonucleases and Ionizing Radiation

5. 1. Introduction

5. 2. Results

5. 2. 1. Effects of ara A on DNA synthesis

5. 2. 2. Effects of ara A on the kinetics of chromatid aberrations of G_2 phase cells

5. 2. 3. Effects of F-ara A on G_2 chromatid aberrations

5. 2. 4. Effects of F-ara A on rejoining of dsb induced by γ-irradiation

5. 2. 5. Effects of ara A on the frequencies of chromatid aberrations induced by RE

5. 2. 6. Influence of T_4 ligase on production of and the potency of ara A in enhancing frequencies of chromosomal aberrations induced by RE

5. 3. Discussion

5. 1. Introduction

Restriction endonuclease (RE)-induced double strand breaks (dsb) possess 5'-phosphoryl and 3'-hydroxyl end-groups, which are thought to be rarely induced by ionizing radiation (Bryant 1988). Because of this "clean" structure, it might be predicted that dsb caused by RE would be directly rejoined by DNA ligase. However, evidence suggests that the repair of RE-induced dsb is more complicated than a simple ligation in that a number of DNA repair inhibitors, e.g., ara C and aphidicolin which affect DNA polymerases and thus may be involved in DNA repair synthesis, have been found to enhance the frequency of RE-induced chromosome aberrations (Natarajan and Obe 1984, Chung et al 1991).

The mechanisms of repair of RE- or radiation-induced dsb in cells are still unclear. Experiments using plasmid DNA have been designed to investigate the capacity of cells to precisely rejoin dsb generated by RE at a specific site in a selectable gene, by identification of the restoration of the gene function (Cox et al 1984, Debenham et al 1988; North et al 1990). It has been found that a proportion of plasmids were mis-rejoined either in plasmid transfected human cells (Cox et al 1984, Debenham et al 1988) or in an *in vitro* system where linearized plasmids were incubated with human cell extracts (North et al 1990). The mis-rejoining always appeared to be associated with the deletion or insertion of a sequence at the initial cutting sites (Ganesh et al 1993; Powell et al 1993). From these studies AT cells were characterised by an increased level of mis-rejoining. The results indicate that simple ligation is unlikely to be the only mechanism by which a cell repairs RE-induced dsb.

Thus a repair pathway requiring DNA resynthesis is likely to be involved in the rejoining of RE-induced dsb in cells. To investigate this notion further, we used 9-β-D-arabinofuranosyladenine (ara A) as a probe to reveal the existence of those repair pathways requiring DNA synthesis

which are usually involved in excision repair (Friedberg 1984) and are thought also to be involved in recombinational repair of radiation-induced DNA strand breaks (Resnick 1976).

Ara A is an analogue of adenosine and deoxyadenosine (Figure 5. 1) that shows potent antivirial and antitumor activities in experimental systems and in the clinic (LePage and Hersh 1972, Cohen 1976). The cytotoxicity of ara A has been associated with its strong and selective inhibitory action on DNA synthesis, protein and RNA synthesis not being affected significantly (Müller et al 1975, 1978, Brockman et al 1980, Plunkett et al 1980). The fluorine derivative of ara A, 9-β-D-arabinifuranosyl-2-fluoroadenine (F-ara A), is also a potent antivirial and antitumor agent (Montgomery and Hewson 1969, Tseng et al 1982). Substitution of hydrogen by fluorine at the 2-carbon position of the purine ring (Figure 5. 1) makes F-ara A resistant to adenosine deaminase, thus overcoming the limited therapeutic activity of ara A because of its rapid deamination by this enzyme (Plunkett and Cohen 1975, Brockman et al 1977).

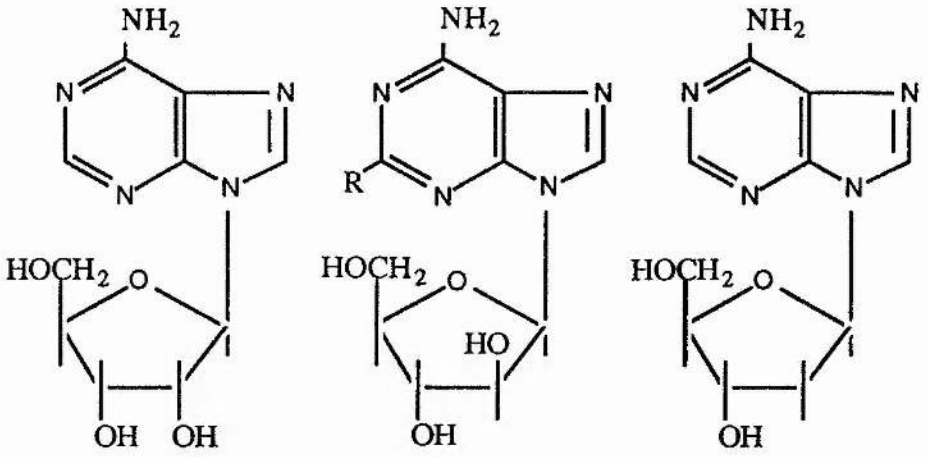

Figure 5. 1. Structures of adenosine, arabinofuranosyladenine (ara A), arabinofuranosyl-2-fluoroadenine (F-ara A) and deoxyadenosine.

Ara A and F-ara A are converted to respective 5'-mono-, di- or triphosphates on entry into cells and the triphosphate of each compound has proved to be the active metabolite responsible for both antivirial and cytotoxic effects (Brink and LePage 1965, Müller et al 1977, Brockman et al 1977, Plunkett et al 1980). Both ara ATP and F-ara ATP have been demonstrated to be competitive inhibitors with respect to dATP for viral DNA-dependent DNA polymerase (Müller et al 1975, Cohen 1976) and eukaryotic polymerases (Müller et al 1975, White et al 1982, Tseng et al 1981, Parker et al 1988). Among the DNA polymerases (pol) in mammalian cells, pol α is affected by ara ATP to the greatest extent (Müller et al 1977, White et al 1982, Parker et al 1988). The inhibition constants (Ki) of ara ATP for pol α is lower than the Michaelis constants (Km) of dATP, indicating a higher affinity of pol α for ara ATP than for the naturally occurring deoxynucleotide (Tseng et al 1982, Parker et al 1988). Pol α was also competitively inhibited by F-ara A, showing an affinity for F-ara A similar

to that for ara ATP (Tseng et al 1982, White et al 1982, Parker et al 1988). However, the increase in activity of pol α during transition from G_1 to S phase was not influenced by incubation of cells with ara A (Müller et al 1977).

Ara ATP also competitively inhibits DNA polymerase β, although the inhibitory effect of ara ATP on pol β is much lower than that on pol α (Tseng et al 1982, White et al 1981, Parker et al 1988). Both lower (Ohno et al 1989) and higher Ki values (Parker et al 1988) of ara A than the Km value of dATP for pol β have been reported. Similarly, pol β has been shown to be much less sensitive to F-ara ATP and Ki value of F-ara A for pol β is higher than the Km value of dATP (Tseng et al 1982, White et al 1981). In contrast to the constant level of pol β throughout the cell cycle, a marginal increase of pol β activity was observed during the transition of cells from G_1 to S phase after incubation with ara A (Müller et al 1977). In addition, pol δ and pol ε (PCNA-independent pol δ) are also inhibited by ara A or F-ara A, athough they are less susceptible to the analogues than pol α (Parker et al 1988, Huang et al 1990).

Other probable target enzymes of ara ATP and F-ara ATP have been suggested to be DNA primase and ribonucleotide reductase. Ara ATP was found to compete with ATP for primase, with approximately 50-fold higher Ki value than Km of ATP for primase (Kuchta and Willhelm 1991). It also incorporates into DNA primers and reduces the ability of DNA pol α to elongate primers (Kuchta and Willhelm 1991). Unlike dATP which acts as a noncompetitive inhibitor of ribonucleotide reductase with respect of four common nucleotides, ara ATP and F-ara ATP competitively inhibit the reduction of ATP or CTP to form ADP or CDP by ribonucleotide reductase for the synthesis of dATP and dCTP (Moor and Cohen 1967, Chang and Cheng 1980, Tseng et al 1982, White et al 1982, Parker et al 1988). Based on these findings, a self-potentiation mechanism was proposed by which the

reduction in the formation of dATP would result in less competition of dATP with ara ATP for polymerases thereby potentiate the inhibition of DNA synthesis (Chang and Cheng 1980, Tseng et al 1982). However, some evidence showed that the intracellular pool size of dNTP was not significantly affect by ara A or F-ara A and therefore the reductase is not likely to be a primary target for these drugs (Moor and Cohen 1967, Müller et al 1977, 1978, Dow et al 1980).

In addition, ara AMP has been found to be incorporated into DNA of mammalian cell nucleus at a very low rate (Müller et al 1975, Kufe et al 1983). The loss of clonogenicity and inhibition of DNA synthesis by the drugs has been shown to correlate with the incorporation of ara AMP into DNA (Plunkett et al 1974; Okura and Yoshida 1978; White et al 1982, TsangLee et al 1980, Kufe et al 1983). Ara AMP residues incorporated into DNA were observed either in internucleotide linkages in DNA or at 3' termini (Müller et al 1975, TsangLee et al 1980). In contrast, F-ara A incorporates into human DNA *in vivo* and *in vitro* at a relatively high rate compared with ara A, and most F-ara AMP residues were found at the 3' termini in DNA strand indicating a chain termination action (Huang et al 1990). The incorporation of F-ara A, rather than inhibition of polymerases, was thought to be the more likely mode of cytotoxicity of the compound (Huang et al 1990). The incorporation of both of the compounds into DNA is diminished when the intracellular pool size of dATP is increased (Müller et al 1977).

Taken together, the mechanisms involved in the inhibition of DNA synthesis by ara A in mammalian cells may be principally due to competitive inhibition of utilization of dATP by DNA polymerases. While for F-ara A, in addition to its competition with dATP for polymerases, it appears to act as a chain terminator once incorporated into DNA.

Ara A in the absence of other treatment is capable of inducing chromosomal damage in human leukocytes (Nichols 1964). The type of chromosomal aberrations observed in metaphase after incubation with ara A for more than one mitotic cell cycle was found to be exclusively breakage, i.e., chromatid deletions and gaps (Nichols 1964). This phenomenon is S phase dependent (as in the case of UV and alkylating agents), and is dissimilar to that induced by ionizing radiation, free radical agents such as bleomycin, and RE, which are all S phase independent in that these DNA damaging agents induce both interchanges and breakage in chromosomes (see review in Chapter 1). A pulse treatment with ara A of G_1 cells before the cells underwent DNA synthesis did not cause chromosomal breakage (Nichols 1964). Observations of Huang and Plunkett (1989) showed that ara A and F-ara A could induce genomic damage by causing deletion of genomic sequences in mutant cells induced by the compounds. They speculated that the incorporated residues of the nucleotide analogs in DNA may lead to a distortion of DNA structure, which may alter DNA replication in the next cell cycle and result in deletion of DNA segments (Huang and Plunkett 1989).

Ara A has been found to enhance the lethality of ionizing radiation in Chinese hamster cells and lead to a removal of the shoulder region from the survival curve of X-irradiated cells (Iliakis 1980, Iliakis et al 1985). This was interpreted in terms of an inhibition of potentially lethal damage repair. Treatment of X-irradiated cells with ara A caused a large increase in the frequencies of anaphase chromosomal abnormalities at the first mitosis following irradiation (Bryant 1980). The disappearance (repair) of chromosome fragments induced by X-irradiation has been found to be inhibited by ara A in G_1 phase cells by using the premature chromosome condensation technique (Iliakis et al 1988). Similar effects of ara A in causing inhibition of the decrease of chromatid breaks with an increasing incubation

time in G_2 phase (interpreted as rejoining of underlying lesions) in normal and AT fibroblasts was reported by Mozdarani and Bryant (1989a) and in Chinese hamster ovary cells (Bryant and Slijepcevic 1993). The potentiation of cell killing and clastogenicity of ionizing radiation by ara A might relate to the inhibition of underlying DNA repair and Bryant and Blöcher (1982) showed that ara A inhibits dsb rejoining in X-irradiated Ehrlich ascites tumour cells.

In this chapter the effects of ara A on the yield of chromosome aberrations resulting from dsb caused by RE were evaluated in two AT lymphoblastoid cell lines (AT-PA and AT-KM) and a normal cell line (N-SW). Restriction enzymes, which produce dsb with different end-structures were used, i.e., *Pvu* II, which cause blunt-ended dsb, and *Pst* I and *Bam*H I, cause 3′ and 5′ cohesive-ended dsb, respectively.

5. 2. Results

5. 2. 1. Effects of ara A on DNA synthesis

The DNA synthesizing capacity of AT-KM and AT-PA lymphoblastoid cells incubated with ara A was measured by the incorporation of ^{3}H-TdR. In order to examine whether the inhibitory effect of ara A decreases with increasing incubation time, the rate of ^{3}H-TdR incorporation during a 1-hour period of incubation was determined following a pre-incubation of cells with ara A for a time interval of 10 min to 4 hours. The relative incorporation of ^{3}H-TdR into DNA in ara A-treated cells in comparison to that of untreated cells was calculated. A suppression of ^{3}H-TdR incorporation was observed for each cell line as a function of ara A concentration after 10 min, 30 min and 4 hours pre-incubation with ara A (Figure 5. 2). AT-PA cells were less sensitive to ara A than AT-KM cells to

the inhibition of DNA synthesis ($p < 0.001$ for 4 h data). Statistically analysis indicated no significant difference ($p > 0.05$) of the relative incorporations in AT-PA cells between 30 min and 4 h pre-incubation with ara A. These results indicated that ara A was active in the cells over the period of incubation.

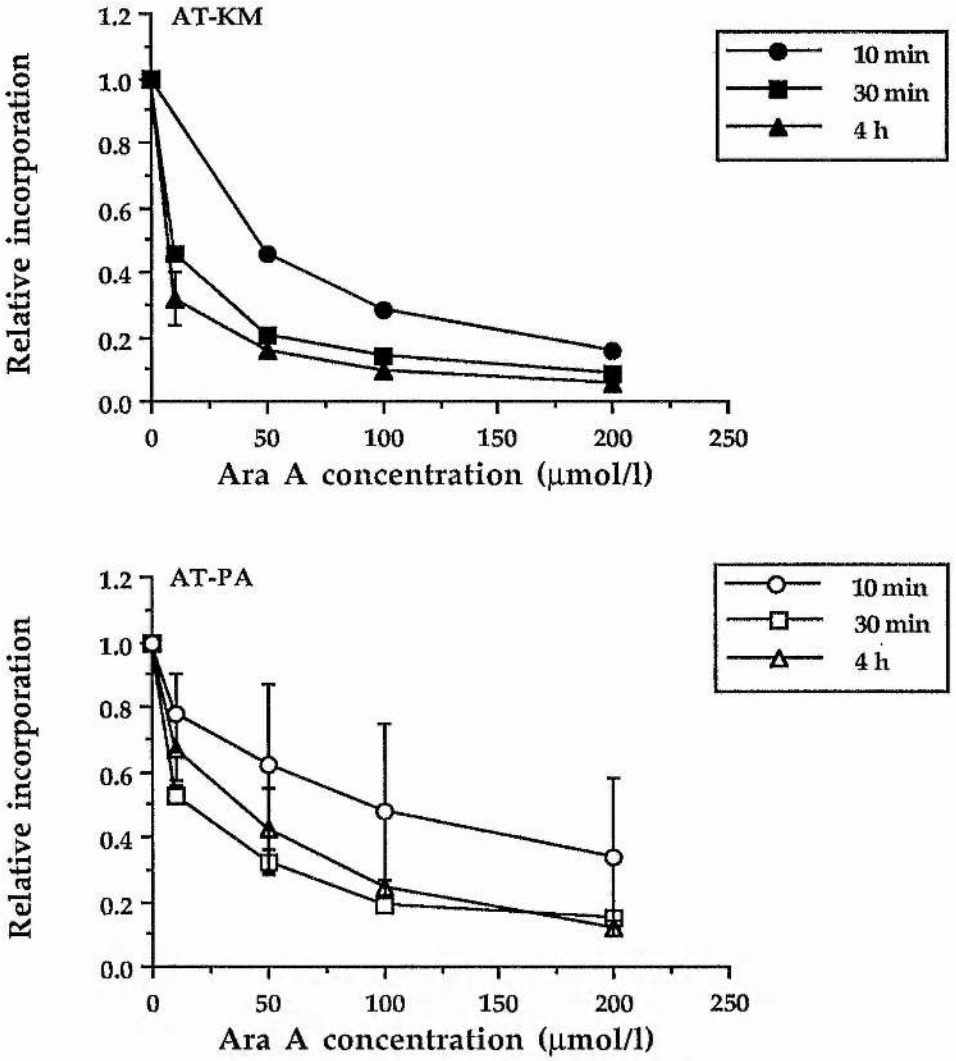

Figure 5. 2. Relative incorporation of ^{3}H-TdR into DNA in AT-KM (upper panel) and AT-PA (lower panel) cells as a function of concentration of ara A. Acid-insoluble radioactivity was determined as described in Materials and Methods (Chapter 2. section 2. 10). Relative incorporation of ^{3}H-TdR was calculated by dividing the dpm of ara A-treated samples by the dpm of parallel control (incubated without ara A). Data were pooled from 2-3 experiments (data for AT-KM cells for 10 and 30 min were from one experiment). Vertical bars represent standard deviations of mean values.

5. 2. 2. Effects of ara A on the kinetics of chromatid aberrations of G_2 phase cells

To investigate the effectiveness of ara A on the kinetics of chromatid aberrations in G_2 phase cells, 2 ml of cells (5×10^5/ml) were pre-incubated with 100 μmol/l of ara A at 37 °C for 30 min before γ-irradiation in the presence of ara A. The cells were further incubated with ara A for various time intervals and colcemid added 3 hours before fixation. Figure 5. 3 shows the kinetics of chromatid aberrations during post-irradiation incubation in the presence of ara A. The background frequencies of aberrations in unirradiated cells are shown in Table 5. 1 and have been subtracted from the original data to give the values in Figure 5. 3. The frequencies of chromatid aberrations induced by γ-irradiation were increased by treatment with 100 μmol/l ara A in the two AT cell lines and the normal cell line. The disappearance of chromatid aberrations occurred both in ara A-treated cells and in untreated cells. Both chromatid deletions and gaps were observed to decline in frequency with incubation time (Table 5. 2). The rate of reduction in numbers of total chromatid aberrations (deletions and gaps) for the AT-KM, AT-PA cell lines was similar to that observed for the N-SW line. No significant difference between the kinetics of disappearance of chromatid aberrations in the presence of ara A and in the absence of ara A was observed.

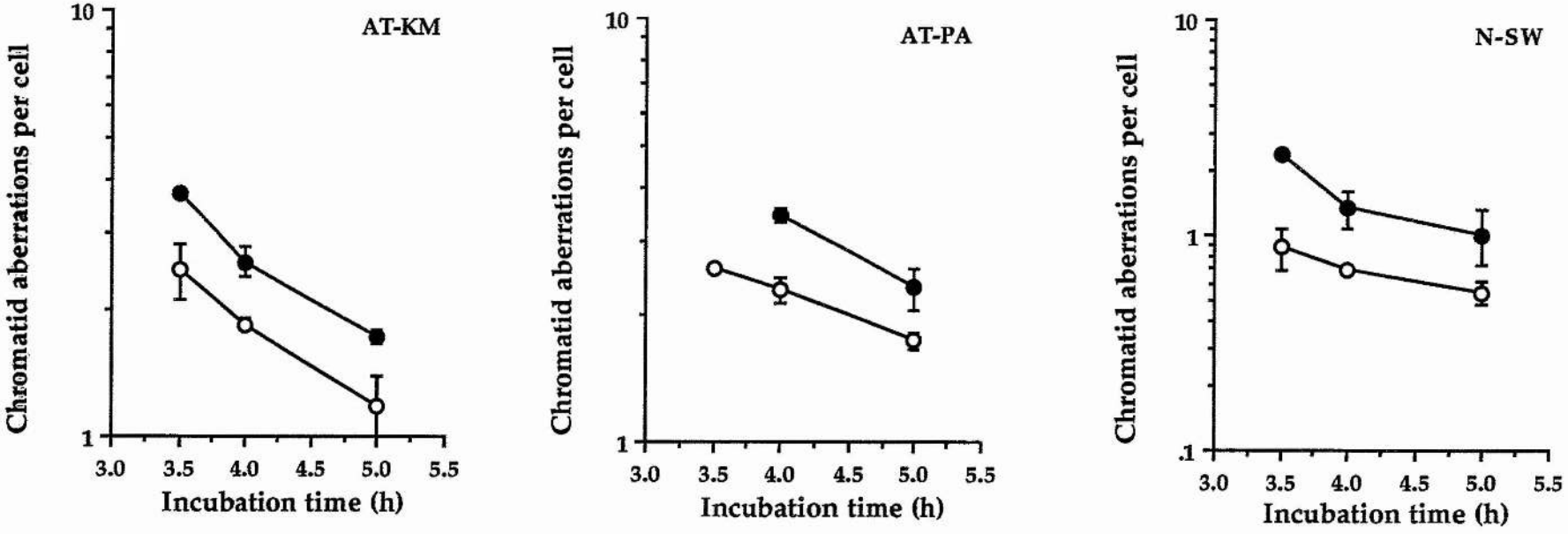

Figure 5. 3. Kinetics of chromatid aberrations induced by 0.3 Gy γ-irradiation in AT-KM, AT-PA and N-SW cells during a 3.5 to 5 hour post-irradiation incubation. Cells were incubated without (opened symbols) or with ara A (100 μmol/l) 30 min before irradiation and were irradiated and incubated in the presence of ara A (solid symbols). Colcemid (0.04 μg/ml) was added 3 hours prior to harvesting. Vertical bars indicate the standard errors of mean values obtained from 2 independent experiments (200 metaphases were scored).

Table 5. 1. Frequencies of chromatid aberrations in unirradiated AT-KM, AT-PA and N-SW cells treated with 100 μmol/l ara A for various time periods. 100 metaphases were scored.

Cell line	ara A (μmol/l)	Incubation time (hour)	Chromatid aberrations per cell
AT-KM	0	4	0.18
	0	5	0.17
	100	3.5	0.48
	100	4	0.44
	100	5	0.49
AT-PA	0	4	0.41
	100	4	0.32
N-SW	0	4	0.10
	100	3.5	0.26
	100	4	0.21

Table 5. 2. Frequencies of chromatid aberrations (CA) per cell induced by 0.3 Gy γ-irradiation in the absence or presence of ara A (100 μmol/l) in AT-PA, AT-KM and N-SW cells (3 hours with colcemid at 0.04 μg/ml). Mean values ± standard errors.

Cell line	γ-ray (Gy)	Ara A (μmol/l)	Incubation time (h)[a]	No. of cells analysed[b]	Chromatid exchange[d]	Chromatid deletion	Chromatid gap	Total CA per cell
AT-KM	0.3	0	3.5	200 (2)	0	1.49 ± 0.01	0.98 ± 0.36	2.47 ± 0.37
	0.3	100	3.5	75 (1)	0	2.77	0.84	3.71
	0.3	0	4	200 (2)	0	1.20 ± 0.13	0.63 ± 0.07	1.83 ± 0.06
	0.3	100	4	162 (2)	0	1.64 ± 0.39	0.93 ± 0.05	2.57 ± 0.21
	0.3	0	5	200 (2)	0	0.61 ± 0.12	0.56 ± 0.09	1.17 ± 0.21
	0.3	100	5	200 (2)	0.005 ± 0.005	0.95 ± 0.05	0.75 ± 0.01	1.71 ± 0.06
AT-PA	0.3	0	3.5	200 (2)	0.007 ± 0.005	1.95 ± 0.12	0.62 ± 0.11	2.57 ± 0.02
	0.3	0	4	200 (2)	0.025 ± 0.005	1.46 ± 0.15	0.80 ± 0.001	2.29 ± 0.16
	0.3	100	4	200 (2)	0.005 ± 0.005	2.28 ± 0.13	1.16 ± 0.007	3.45 ± 0.13
	0.3	0	5	200 (2)	0.015 ± 0.005	1.06 ± 0.06	0.65 ± 0.02	1.73 ± 0.07
	0.3	100	5	200 (2)	0.020 ± 0	1.46 ± 0.09	0.84 ± 0.15	2.31 ± 0.25
N-SW	0.3	0	3.5	200 (2)	0	0.47 ± 0.09	0.41 ± 0.10	0.88 ± 0.19
	0.3	100	3.5	100 (1)	0	2.06	0.34	2.40
	0.3	0	4	200 (2)	0	0.35 ± 0.05	0.34 ± 0.03	0.69 ± 0.02
	0.3	100	4	200 (2)	0	1.03 ± 0.03	0.32± 0.24	1.35 ± 0.27
	0.3	0	5	200 (2)	0.015 ± 0.01	0.23 ± 0.07	0.29 ± 0.02	0.54 ± 0.07
	0.3	100	5	200 (2)	0	0.64 ± 0.19	0.37 ± 0.10	1.01 ± 0.29

[a], [b] and [d] have the same meanings as in Table 3.1.

5. 2. 3. Effects of F-ara A on G_2 chromatid aberrations

It has been found that the deamination of ara A by adenosine deaminase in human lymphocytes results in a loss of the ability of the drug to inhibit the repair of chromosomal damage induced by ionizing radiation (MacLeod and Bryant 1992). To determine whether the disappearance of chromatid aberrations in ara A-treated cells is a result of the degradation of ara A in the lymphoblastoid cells, F-ara A which is resistant to adenosine deaminase (Plunkett et al 1980) was applied at concentrations of either 100 or 1000 μmol/l. Table 5. 3 shows that F-ara A similarly enhanced the frequencies of chromatid aberrations in irradiated cells. As in the case of ara A, a longer period (5 h) of post-irradiation incubation led to an approximately 2-fold reduction in the aberrations when compared to the short period (3.5 h) of incubation for all the cell lines treated with 100 μmol/l F-ara A. Treatment with a high concentration of F-ara A (1000 μmol/l) resulted in a remarkably high level of background aberrations in unirradiated cells, while the absolute frequency of aberrations, which are obtained by subtracting the value in unirradiated cells from that in irradiated cells, was similar to that obtained from 100 μmol/l F-ara A-treated cells. Therefore, there was no apparent difference between the potentiation effects of F-ara A and of ara A in the kinetics of G_2 chromatid aberrations in the cell lines used.

Table 5. 3. The effects of F-ara A on the frequencies of chromatid aberrations (CA) in γ-irradiated AT and normal lymphoblastoid cells at 3.5 or 5 hour post-irradiation incubation time. Data were from a single experiment and 100 metaphases were scored for each sample.

Cell line	F-ara A (μmol/l)	γ-ray (Gy)	Incubation time (h)[a]	Chromatid exchange	Chromatid deletion	Chromatid gap	Total CA per cell	CA per cell (bkg subtracted)[b]
AT-PA	100	0	5	0	0.13	0.29	0.42	---
	100	0.3	3.5	0.01	2.52	1.09	3.61	3.20
	100	0.3	5	0.01	1.36	0.49	1.85	1.43
	1000	0	5	0	1.04	0.68	1.72	---
	1000	0.3	5	0	2.76	0.75	3.51	1.79
AT-KM	100	0	5	0	0.08	0.36	0.44	---
	100	0.3	3.5	0	1.84	1.68	3.52	3.06
	100	0.3	5	0	0.94	0.97	1.91	1.47
N-SW	100	0	5	0	0.09	0.15	0.24	---
	100	0.3	3.5	0	0.88	0.50	1.38	1.14
	100	0.3	5	0	0.46	0.30	0.76	0.52

[a]: Incubation time after irradiation in the presence of F-ara A which was added to cell cultures 30 min before irradiation.

[b]: Background (bkg) chromatid aberrations of F-ara A treated and unirradiated cells.

5. 2. 4. Effects of F-ara A on rejoining of dsb induced by γ-irradiation

The production of dsb immediately after 20 Gy γ-irradiation in the presence of F-ara A (100 or 1000 μmol/l) was found to be similar to those observed in the absence of F-ara A in AT-PA and N-SW cells as measured with non-denaturing (pH 9.6) filter elution method (Chapter 2 section 2.9). One exception was the fraction of DNA eluted in N-SW cells treated with 1000 μmol/l of F-ara A, which increased in the unirradiated control cells and was reduced in the irradiated cells (Figure 5. 4).

Rejoining of dsb induced by 20 Gy γ-rays during post-irradiation incubation in the presence or absence of F-ara A is shown in Figure 5. 5. The results show that F-ara A (100 μmol/l) does not inhibit dsb rejoining in AT-PA and N-SW cells. In one experiment, cells were treated with 1000 μmol/l of F-ara A and the extent of dsb rejoining at 2 hour post-irradiation incubation was found to be over 100% for N-SW cells, and 94.9% for AT-PA which was similar to that in 100 μmol/l F-ara A-treated AT-PA cells (95.3%). These results indicate that F-ara A at as high concentration as 1000 μmol/l is still not able to inhibit dsb rejoining in these cell lines.

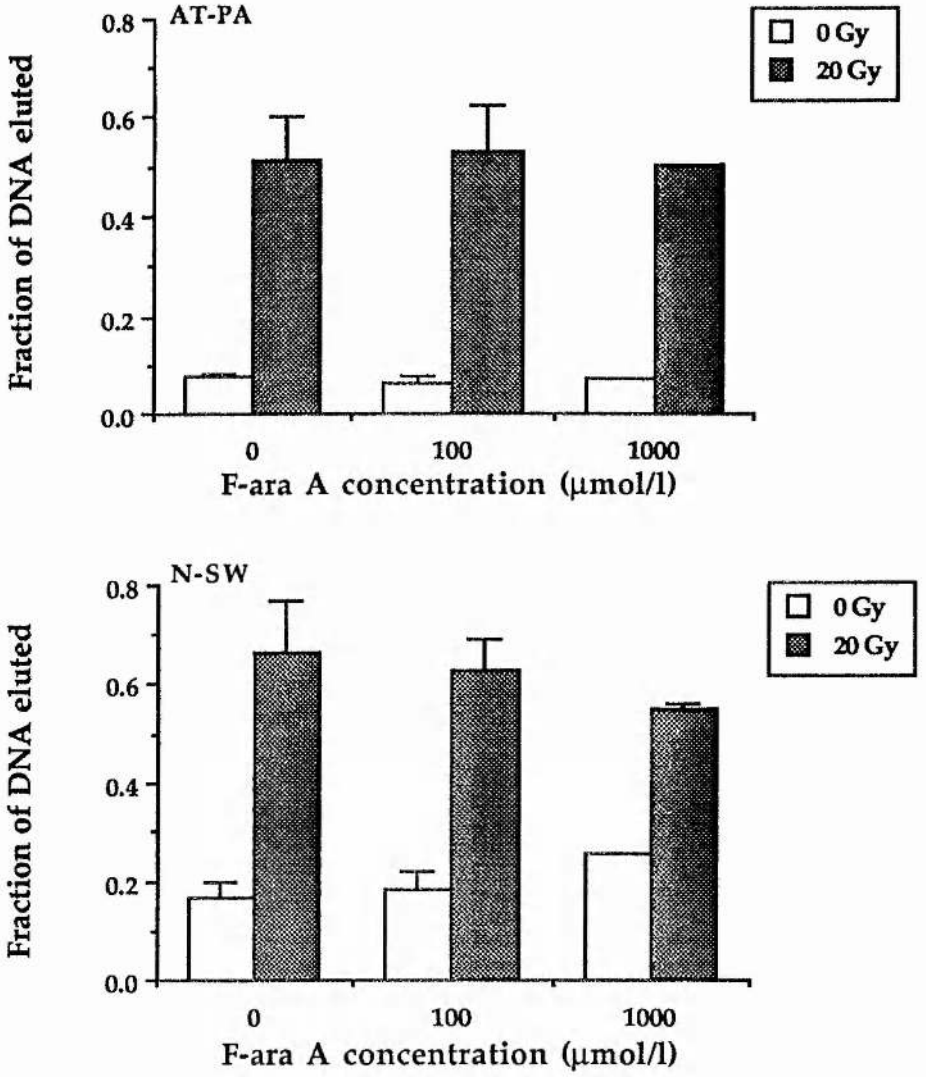

Figure 5. 4. Induction of dsb immediately after 20 Gy γ-irradiation in AT-PA (upper panel) and N-SW (lower panel) cells treated with F-ara A. Vertical bars represent standard errors of mean values obtained from at least 3 (for 0 and 100 µmol/l of F-ara A) and 2 (for 1000 µmol/l of F-ara A) independent experiments.

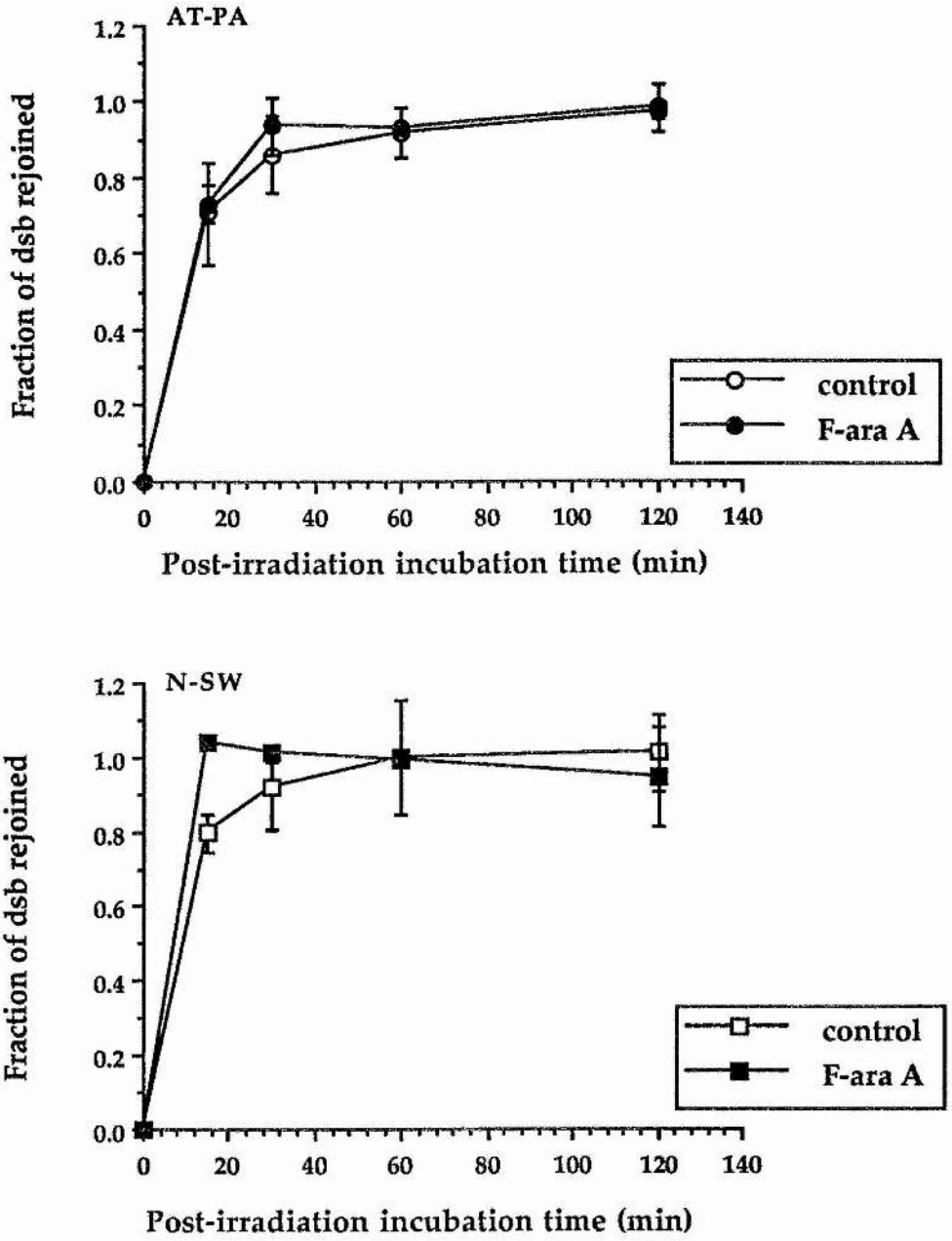

Figure 5. 5. Rejoining of dsb in AT-PA (upper panel) and N-SW (lower panel) cells treated with 100 μmol/l of F-ara A and irradiated by 20 Gy γ-rays. The fraction of dsb rejoined was calculated by the formula described in Chapter 2 (section 2.9). Vertical bars indicate the standard errors of mean values obtained from at least 3 independent experiments.

5. 2. 5. Effects of ara A on the frequencies of chromatid aberrations induced by RE

The effect of ara A on the frequency of chromatid aberrations induced by *Pvu* II, *Pst* I and *Bam*H I is illustrated in figure 5. 6. When different RE were compared in untreated cells, *Pvu* II appeared most clastogenic, while *Pst* I and *Bam*H I induced lower numbers of chromosome aberrations in all the cell lines. Both AT-KM and AT-PA cells exhibited higher frequencies of chromatid aberrations than N-SW cells after *Pvu* II, *Pst* I and *Bam*H I treatment. The application of 100 μmol/l of ara A resulted in a marked enhancement in the yield of chromatid damage induced by *Pvu* II and *Pst* I, whereas the yield of chromatid aberrations induced by *Bam*H I did not appear to be significantly influenced by ara A treatment in any of the cell lines.

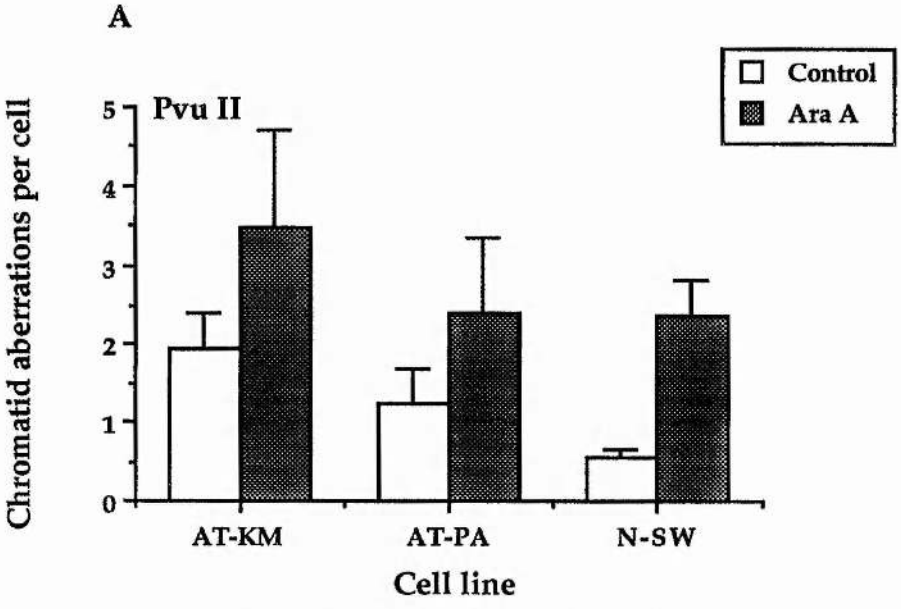

Figure 5. 6 (A). The effects of ara A (100 μmol/l) on the frequencies of chromatid aberrations induced by *Pvu* II at 50 units/ml in SLO (0.06 units/ml) porated AT-KM, AT-PA and N-SW cells after 5 hours post-treatment incubation. Controls were porated with SLO in the presence of HBSS/BAS in the volume equal to that of RE. The number of experiments is shown in Table 5. 4 to 5. 6.

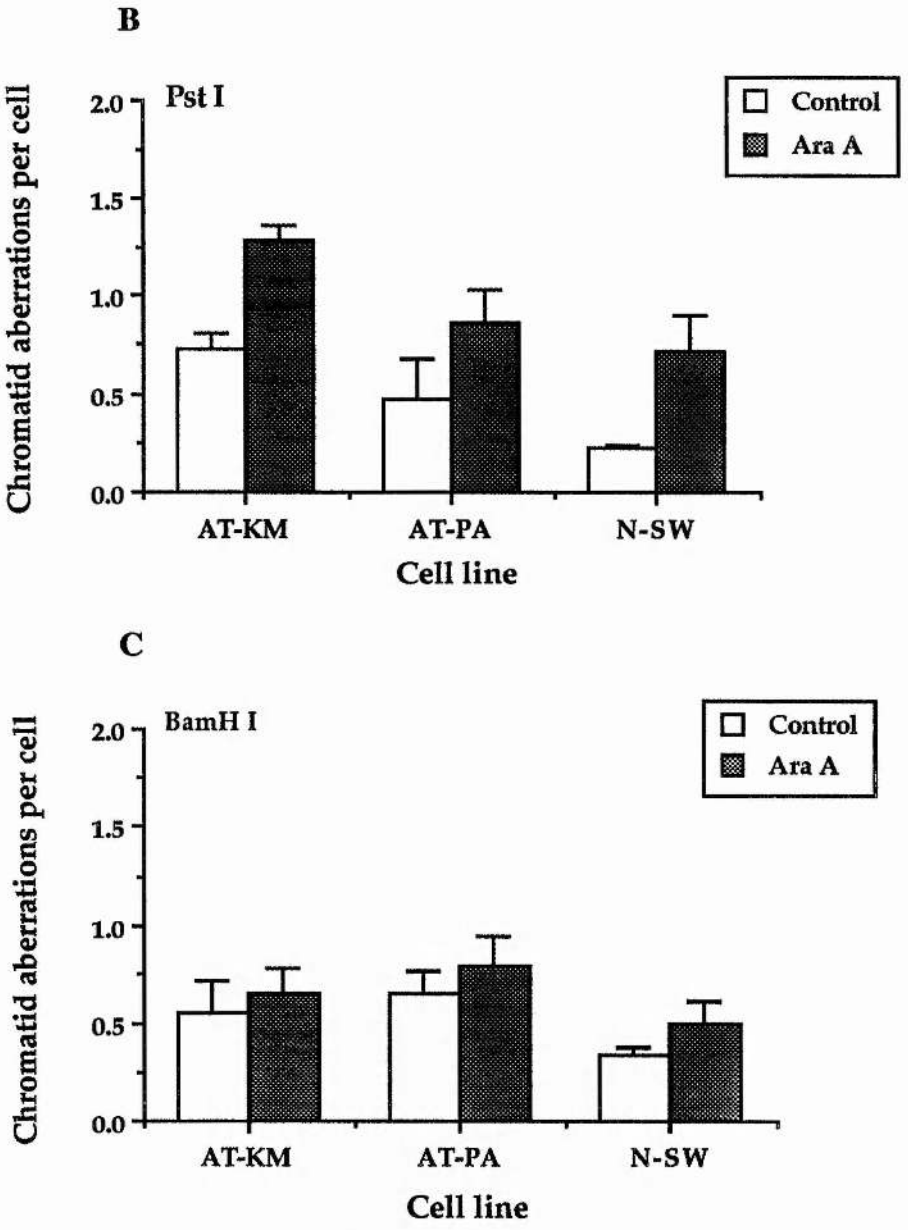

Figure 5. 6 (continue). The effects of ara A (100 μmol/l) on the frequencies of chromatid aberrations induced by *Pst* I (B) and *Bam*H I (C) at 50 units/ml in SLO (0.06 units/ml) porated AT-KM, AT-PA and N-SW cells after 5 hours post-treatment incubation. Controls were porated with SLO in the presence of HBSS/BAS in the volume equal to that of RE. The number of experiments is shown in Table 5. 4 to 5. 6.

The enhancement by ara A of chromatid aberration frequencies induced by RE are shown in detail in Tables 5. 4 to 5. 6 for AT-KM, AT-PA and N-SW cell line, respectively.

Table 5. 4. Chromatid aberrations (CA) in SLO porated AT-KM cells treated with 50 units/ml of RE and ara A (100 μmol/l). Mean values ± standard errors.

Treatment	No. of cells analyzed[a]	% aberrant cells	Chromatid exchange per cell	Chromatid deletion per cell	Chromatid gap per cell	Total CA per cell
Control	200 (2)	20.5 ± 1.5	0	0.11 ± 0.05	0.12 ± 0.03	0.23 ± 0.01
Control + ara A	200 (2)	35.5 ± 8.5	0	0.24 ± 0.08	0.26 ± 0.06	0.50 ± 0.14
Pvu II	300 (3)	38.0 ± 5.2	0.02 ± 0.02	1.22 ± 0.28	0.69 ± 0.11	1.96 ± 0.44
Pvu II + ara A	200 (2)	54.5 ± 10.5	0.04 ± 0.01	2.14 ± 0.68	1.31 ± 0.53	3.49 ± 1.23
Pst I	200 (2)	36.0 ± 1.0	0.01 ± 0.01	0.39 ± 0.04	0.32 ± 0.04	0.72 ± 0.08
Pst I + ara A	200 (2)	48.0 ± 7.0	0.02 ± 0.005	0.68 ± 0.16	0.58 ± 0.08	1.29 ± 0.08
*Bam*H I	300 (3)	27.6 ± 10.9	0	0.25 ± 0.05	0.29 ± 0.15	0.55 ± 0.17
*Bam*H I + ara A	300 (3)	35.7 ± 10.1	0.007 ± 0.009	0.33 ± 0.12	0.35 ± 0.08	0.65 ± 0.13

[a]: Number of independent experiments is shown in parenthesis.

Table 5. 5. Chromatid aberrations (CA) in SLO porated AT-PA cells treated with 50 units/ml of RE and ara A (100 μmol/l). Mean values ± standard errors.

Treatment	No. of cells analyzed[a]	% aberrant cells	Chromatid exchange per cell	Chromatid deletion per cell	Chromatid gap per cell	Total CA per cell
Control	300 (3)	18.8 ± 0	0	0.08 ± 0.04	0.14± 0.05	0.22 ± 0.005
Control + ara A	200 (2)	25.0 ± 1.0	0	0.22± 0.01	0.14 ± 0.01	0.36 ± 0.005
Pvu II	400 (4)	44.2 ± 8.7	0	0.52 ± 0.02	0.73 ± 0.02	1.25 ± 0.46
Pvu II + ara A	300 (3)	50.5 ± 12.5	0	1.31± 0.59	1.09 ± 0.36	2.40 ± 0.95
Pst I	200 (2)	36.9 ± 12.4	0	0.18 ± 0.08	0.29 ± 0.12	0.47 ± 0.20
Pst I + ara A	200 (2)	53.5 ± 11.5	0	0.35± 0.09	0.50± 0.08	0.85 ± 0.17
*Bam*H I	300 (3)	43.3 ± 8.7	0	0.20± 0.07	0.45 ± 0.10	0.65 ± 0.12
*Bam*H I + ara A	300 (3)	50.7 ± 11.7	0	0.36± 0.08	0.43 ± 0.14	0.79 ± 0.15

[a]: Number of independent experiments is shown in parenthesis.

Table 5. 6. Chromatid aberrations (CA) in SLO porated N-SW cells treated with 50 units/ml of RE and ara A (100 μmol/l). Mean values ± standard errors.

Treatment	No. of cells analyzed[a]	% aberrant cells	Chromatid exchange per cell	Chromatid deletion per cell	Chromatid gap per cell	Total CA per cell
Control	400 (4)	12.2 ± 5.5	0	0.04 ± 0.02	0.09 ± 0.05	0.13 ± 0.06
Control + ara A	300 (3)	30.0 ± 7.8	0	0.24 ± 0.04	0.17 ± 0.06	0.41 ± 0.11
Pvu II	300 (3)	22.3 ± 1.7	0.003 ± 0.003	0.27 ± 0.05	0.27± 0.07	0.54 ± 0.11
Pvu II + ara A	300 (3)	28.5 ± 6.4	0.005 ± 0.007	1.29 ± 0.43	1.02 ± 0.09	2.32 ± 0.52
Pst I	200 (2)	17.5 ± 3.5	0.005 ± 0.005	0.07 ± 0.01	0.14 ± 0.05	0.22 ± 0.02
Pst I + ara A	200 (2)	31.5 ± 2.5	0	0.36 ± 0.05	0.35 ± 0.12	0.71 ± 0.17
*Bam*H I	300 (3)	23.0 ± 4.6	0	0.14 ± 0.02	0.21 ± 0.06	0.35 ± 0.04
*Bam*H I + ara A	300 (3)	33.3 ± 8.1	0	0.25 ± 0.06	0.25 ± 0.05	0.50 ± 0.12

[a]: Number of independent experiments is shown in parenthesis.

In all 3 cell lines, incubation with ara A alone for 5 hours caused an increase of the background aberrations in the control cells (Tables 5. 4, 5. 5 and 5. 6). Combination treatment with RE and ara A of AT-KM cells resulted in an increase in deletions as well as of gaps induced by *Pvu* II and *Pst* I, but not of those induced by *Bam*H I (Table 5. 4). Chromatid exchanges were slightly increased by ara A treatment in AT-KM cells (Table 5. 4). Similar results were found in AT-PA cells except that no exchange was scored (Table 5. 5). In the normal N-SW cell line (Table 5. 6), although *Pvu* II and *Pst* I caused much lower frequencies of chromatid aberrations in the absence of ara A when compared with AT-KM or AT-PA cell lines, the chromatid deletions and gaps were dramatically increased in the presence of ara A. By comparison, ara A had only a minor effect on chromatid aberrations induced by *Bam*H I in N-SW cells

The ara A enhancement ratio was calculated as a ratio between the yields of aberrations of ara A-treated cells and those of untreated cells. The ratios are shown in Table 5. 7. After *Pvu* II treatment, the enhancement ratio is highest for N-SW cells while the other two AT cell lines show comparable values. Similar results are found for *Pst* I and the ratios are comparable with those for *Pvu* II in each of the cell lines. The ratios for *Bam*H I are lowest when compared to those for *Pvu* II or *Pst* I in each cell lines. The ratio for γ-irradiation is also highest for N-SW cells when compared to those for the two AT cell lines, while all ratios for γ-rays are lower than those for RE treatment (with the exception of those for *Bam*H I).

Table 5. 7. Ara A enhancement ratio for chromatid aberrations in RE-treated and γ-ray irradiated cells.

Cell line	*Pvu* II	*Pst* I	*Bam*H I	Gamma-ray*
AT-KM	1.78	1.78	1.17	1.27
AT-PA	1.94	1.82	1.21	1.51
N-SW	4.38	3.18	1.44	1.96

* 4-hour post-irradiation (0.3 Gy) incubation.

5. 2. 6. Influence of T_4 ligase on production of and the potency of ara A in enhancing frequencies of chromosomal aberrations induced by RE

In order to explore the possible importance of a ligation step in the repair of RE-induced dsb, the T_4 ligase was co-porated into cells with RE in the presence or absence of ara A, and the frequencies of chromatid aberrations at 5 hour incubation time examined. T_4 ligase (1 unit/μl) in stock solution was purified by ultrafiltration as described in Materials and Methods (Chapter 2 section 2. 2) and concentrated to 10 units/μl in HBSS/BSA before use. An *in vitro* test with RE-linearized pBR 322 indicated that the ligase was fully active following purification (data not shown).

As shown in Table 5. 8, T_4 ligase, once porated into cells, was neither capable of reducing the frequencies of chromatid aberrations nor abolishing the potentiation effects of ara A. Instead, it appeared to increase the number of chromatid aberrations (both deletions and gaps), particularly in N-SW cells treated with RE either in the presence or absence of ara A.

Table 5. 8. Influence of T_4 ligase on the frequencies of chromatid aberrations induced by *Pvu* II (100 units/ml) in SLO (0.06 units/ml) porated cells.

Cell line	T_4 ligase (units/ml)	ara A (μmol/l)	% aberrent cells[a]	Chromatid exchange per cell	Chromatid deletion per cell	Chromatid gap per cell	Total chromatid aberrations per cell
N-SW	0	0	20 (100)	0.01	0.17	0.21	0.39
	0	100	34 (100)	0	0.78	0.18	0.96
	50	0	20 (100)	0	0.37	0.21	0.59
	150	0	20.5 (200)	0.001	0.35	0.25	0.61
	150	100	32 (100)	0	0.80	0.32	1.12
AT-KM	0	0	30 (100)	0.01	0.61	0.23	0.85
	0	100	42 (100)	0	0.46	0.60	1.06
	150	0	41 (100)	0	0.55	0.32	0.87
	150	100	44 (50)	0	0.78	0.46	1.24

[a]: Number of metaphases scored in the parenthesis.

5. 3. Discussion

Potentiation of the clastogenic effects of ionizing radiation by ara A

Ara A is known as a potent inhibitor of DNA synthesis and exerts a potentiating effect on radiation-induced cell killing and chromosomal damage probably via an inhibition of DNA replication required in a DNA repair pathway. The present study has focused on the effects of ara A on the induction and repair of G_2 chromatid aberrations induced by γ-rays or RE. The advantage of using G_2 cells is that the proliferative cytoxicity and clastogenicity of ara A, particularly on S phase cells, may be minimised. During S phase, cells have been shown to be much more susceptible to the immediate lethal consequences of ara A and F-ara A (Dow et al 1980). Both compounds prevent the progression of cells through S phase, while having little effect on the arrest of cells in G_2 phase (Dow et al 1980).

A process which requires DNA synthesis for the repair of DNA damage occurring in G_2 phase has been suggested by Palitti et al (1983). In their studies, the potentiation of chromosomal aberrations by DNA repair inhibitors, hydroxyurea (HU) and aphidicolin, have been observed in CHO cells treated with S phase dependent agents (i.e., UV, 4NQO and MMC) in G_1 phase and challenged in G_2 phase with the inhibitors. Since both HU and aphidicolin are known to inhibit DNA synthesis (Young and Hodas 1964, Ikegami et al 1978), these results indicate that DNA synthesis is required for the repair of DNA damage in G_2 phase cells. This hypothesis may partly provide a basis for the inhibitory effects of ara A on DNA synthesis.

Following G_2 irradiation, the cells treated with ara A yield more chromatid aberrations, both deletions and gaps, than untreated cells (Figure 5. 3 and Table 5. 3). Ara A alone at 100 μmol/l caused a low frequency of chromatid aberrations and this was irrespective of the time of incubation (3.5 to 5 hours) (Table 5. 1). The extent of potentiation of aberration

frequencies by ara A in both AT-KM and AT-PA cell lines was not significantly different from that in the normal N-SW cell line, although the AT cell lines were about twice as sensitive to γ-irradiation as normal cells at G_2 (Figure 5. 3). The kinetics of chromatid aberrations following the exposure of cells to γ-rays in the absence of ara A declined with increasing time between irradiation and fixation in all cell lines (Figure 5. 3). A similar reduction in chromatid aberrations was observed in ara A-treated cells, despite the fact that levels of aberrations were elevated over those observed in the absence of ara A (Figure 5. 3). This result was unexpected and inconsistent with the observations of the complete inhibition of chromosomal repair by ara A in AT and normal fibroblasts (Mozdarani and Bryant 1989a, b). It is possible that degradation of ara A by adenine deaminase occurs in lymphoblastoid cells thereby reducing the effects of ara A. In this regard, F-ara A, which is resistant to adenine deaminase, was used in the present study. Results obtained indicated a similar decrease in chromatid aberrations to that observed in the case of ara A (Table 5. 4). This finding implies that the inability of ara A to completely inhibit the chromosomal repair may not be due to the deamination of the drug. Another possibility may be related to the different responses of the cell lines to ara A. Indeed, the human lymphoblastoid cell lines did show a difference in their response to treatment with ara A, with ara A potentiating the frequency of radiation-induced chromatid gaps as well as chromatid deletions (Table 5. 2), while in fibroblasts the number of gaps was not significantly altered by ara A treatment (Mozdarani and Bryant 1989a).

The consistently increased level of radiation-induced chromatid aberrations associated with ara A implies that ara A may increase the proportion of unrepaired DNA damage which is expressed as chromatid aberrations in metaphase following irradiation. However, the rejoining of dsb induced by γ-ray in AT-PA and N-SW cells was not affected by F-ara A

(100 μmol/l) (Figure 5. 5). Even F-ara A at a high concentration (1000 μmol/l) did not impair dsb rejoining, despite a dramatic increase in the level of chromosomal damage (Table 5. 3). This is again contrary to previous findings that ara A acts to inhibit dsb rejoining in Ehrlich ascites tumour cells as measured by the DNA unwinding method (Bryant and Blöcher 1982). It has been suggested that neutral filter elution may detect different types of dsb from those detected by the DNA unwinding technique (Costa and Bryant 1990b). With neutral elution, more than 80% of dsb were repaired in Ehrlich ascites tumour cells after 30 Gy X-irradiation in the presence of 400 μmol/l of ara A. At this concentration of ara A the rejoining of dsb induced by the same dose of X-rays, when detected by the unwinding technique, was completely inhibited (Costa and Bryant 1990b).

The mechanism by which ara A potentiates radiation effects on cells is presently unclear, although (as mentioned above) there is evidence that ara A inhibits the repair of dsb (Bryant and Blöcher 1982). The structural alterations at the chromosomal and DNA level caused by ara A or F-ara A has been suggested to be the result of the replicative incorporation of these arabinosyl nucleotides into DNA (Nichols 1964, Huang and Plunkett 1989). The incorporation, however, does not appear to fully explain the mechanism by which ara A inhibits the repair of DNA lesions induced by ionizing radiation. Ara A is incorporated into DNA in a very low proportion; 1 molecule in 8000 molecules of naturally occurring adenine as estimated by Müller et al (1975). Since the repair synthesis for repairing radiation-induced lesions was proposed to involve only a few nucleotide residues (Regan and Setlow 1974), ara A or F-ara A is unlikely to cause DNA structural distortion or to terminate the strand elongation by incorporation into the newly synthesised patch of DNA.

From the kinetics of G_2 chromosomal aberrations (Figure 5. 3), two components of DNA damage that are expressed as chromosomal aberrations

may be postulated. The first component of damage may be repaired and exhibits a decrease with time; the other component of damage may not be repaired and constitute the higher portion of aberrations at each time point as seen in AT cells when compared with normal cells, and in ara A treated cells when compared with untreated cells. The dsb may be the major DNA lesion that constitute this second component. Ara A increases this component of damage probably by its inhibitory effects on DNA repair synthesis.

Potentiation of clastogenic effects of restriction endonucleases-induced dsb by ara A

The results presented here demonstrate that the DNA replication inhibitor ara A potentiates the frequency of chromatid aberrations which originate from dsb generated by RE. This potentiation indicates that the repair of RE-induced dsb in chromatid DNA involves a mechanism which requires DNA synthesis. The results with ara A are consistent with those using ara C, which was similarly found to increase the frequencies of chromosomal aberrations induced by RE (Natarajan and Obe 1984; Chung et al 1991). Ara C increased the frequencies of chromosomal aberrations induced by both *Alu* I (generates blunt-ended dsb) and *Sau*3A I (generates cohesive-ended dsb), while another inhibitor of DNA replication, aphidicolin, increased the level of aberrations caused by *Sau*3A I but had no effect on *Alu* I-induced aberrations, indicating a different repair pathway may exist for the repair of dsb with different end structures (Chung et al 1991). A plausible explanation for the potentiation by DNA synthesis inhibitors of chromosomal aberrations induced by RE is that DNA synthesis occurs following an end degradation, probably by an exonuclease, of RE-induced dsb. Thus, ara A is likely to alter the steps following degradation and prior to ligation. It is possible that only a portion of RE-induced dsb are

subject to end-degradation, while the other portion may be rejoined simply by ligase.

AT cells (AT-KM and AT-PA) showed elevated frequencies of chromatid aberrations over those in normal (N-SW) cells after treatment with RE inducing either blunt- (*Pvu* II) or cohesive-ended (*Pst* I and *Bam*H I) dsb. This suggests a defect in dsb processing in AT cells as we have previously proposed (Chapter 3 and Liu and Bryant 1993). Increased frequencies of chromatid aberrations were observed after ara A treatment in both AT and normal cells treated with *Pvu* II and *Pst* I, although ara A has been reported to be unable to increase the yield of dsb induced in CHO cells by treatment with 200 units/ml of *Pvu* II, as measured with neutral filter elution (Costa and Bryant 1991b). AT cells were shown to be influenced by ara A to a lesser extent than normal cells (Table 5. 7). The difference in response to ara A between AT and normal cells is unlikely to be a reflection of a reduced level of degradation at the ends of dsb in AT cells, since AT cells have been reported to be more sensitive to strand end exposure, suggested by the results indicating a disequilibrium between ligation and exonuclease digestion of double strand (Cox et al 1984) and less protection of the ends of dsb in AT cells (North et al 1990). One possible explanation for these findings may be that the repair of dsb by an "excision" pathway is relatively efficient in normal cells, therefore the effect of its inhibition is more obvious when repair is impaired by ara A. In AT cells however, the end-degradation may be far too extensive to be managed by the excision repair, thus making AT cells apparently insensitive to ara A.

The nature of the defect in DNA processing in AT cells has proved difficult to elucidate. Paterson et al (1976) has suggested that AT cells are defective in an initiation, which is mediated by an endonuclease, of an excision-type repair to deal with base damage. AT cells, however, have been found to be specially hypersensitive to those DNA damaging agents which

create strand breaks in DNA rather than those only inducing base damage (Shiloh et al 1985). The defective steps of repair in AT cells therefore, seem to relate to the later stage, after strand disruption. Inoue et al (1977) have demonstrated that AT cells are deficient in the action of a "primer activating enzyme" which functions to modify the radiation-induced "dirty" 3′ ends of gaps, allowing DNA polymerisation to subsequently fill the gap. This mechanism cannot be operative on RE-induced dsb since the termini have 5′-phosphoryl and 3′-hydroxyl groups which do not need to be cleaned before ligation.

A number of enzymes which may be involved in dsb rejoining, e.g., DNA ligase I and II (Willis and Lindahl 1987), polymerase α and β (Bertazzoni et al 1978), have been reported normal in AT cells. The synthesis of poly(ADP-ribose) following γ-irradiation was found to be deficient in two AT cell lines as observed by Edwards and Taylor (1980). Poly(ADP-ribose) polymerase is thought to play a role in strand break rejoining (Ahnström and Ljungman 1988) through binding to DNA breaks following its activation by the breaks, and of catalysing the polymerization of ADP-ribose units on chromosomal proteins, including histone H1 and on the enzyme itself (Murcia et al 1988). As a result of the accumulation of negative charge in chromosomal proteins, a local decondensation of chromatin occurs (Murcia et al 1988), making DNA more accessible and allowing repair enzymes to rejoin the breaks. The defect in the activation of poly(ADP-ribose) polymerase by irradiation, however, was not uniformly demonstrated in all AT cell lines (Zwelling et al 1983). AT cells have been found to be more sensitive to the inhibitor of Top II, a phenomena which was associated with an abnormal overproduction of the enzyme (Smith and Makinson 1989). A reduced activity of Top II has also been reported in one AT cell line (Singh et al 1988).

A reduction of RE-induced chromosomal damage by T_4 ligase as assayed by the micronucleus technique in CHO cells was reported by Bryant and Johnston (1992). Durante et al (1991) reported that concomitant treatment of cells with T_4 ligase considerably reduced the lethal effects of *Pvu* II on C3H10T1/2 cells. Co-administration of T_4 ligase with *Pvu* II in the present study, however, failed to reduce the chromatid aberrations caused by the enzyme. Nor was T_4 ligase able to reverse the potentiation effects of ara A in the human lymphoblastoid cell lines. Two explanations may be made. First, the ligase activity in the cells may already be sufficient so that the addition of exogenous ligase has little effect. Secondly, T_4 ligase itself plays but a minor role in the competition between ligation and degradation. These explanations, however, can not fully account for the effect of T_4 ligase in *increasing* chromosomal aberrations in RE and ara A treated cells. The significance of this observation is not presently clear. One possible explanation is that T_4 ligase may be not identical to the functional ligase in human cells. Evidence has shown that an inhibitor of ligase I has no effect on T_4 ligase or ligase II (Yang et al 1992).

It is of interest to note that the effects of ara A are different for dsb with different end-structures. Blunt-ended dsb induced by *Pvu* II and 3'-overhang cohesive dsb induced by *Pst* I show similar sensitivities to ara A, although *Pvu* II yields approximately 2-fold more aberrations than *Pst* I in AT and normal cell lines. By contrast, 5'-overhang cohesive-ended dsb induced by *Bam*H I seem to be insensitive to ara A. This implies that dsb with different end-structures may undergo different repair pathways or different extents of degradation. The difference in clastogenic effects between blunt-ended and cohesive-ended dsb may be due in part to different efficiencies of DNA rejoining by DNA ligase. It seems plausible that the major proportion of RE-induced dsb which are directly and rapidly rejoined by DNA ligase are those with cohesive-ends (Bryant 1985, Costa and Bryant

1989). However, the end-structure of RE-induced dsb, e.g., the degree of overlapping and the direction of breaks, may influence the speed or efficiency of the ligation. A limited end-degradation by an excision repair mechanism may be more likely to occur when the broken-ends are left unrepaired for relatively long time. Blunt-ended dsb induced by *Pvu* II, as well as 3' cohesive-ended dsb caused by *Pst* I, seem to be more susceptible to this end-degradation and are thereby more sensitive to the treatment with ara A.

The different response to ara A of 3′- and 5′- overhang cohesive-ended dsb suggests that the degradation may not be random in direction. One possible explanation may be that 3′-5′ exonuclease activity is dominant. If this is the case, the removal of sequence from a 3′-overhang cutting site might potentially result in a blunt-ended dsb which is likely to reduce the rate of rejoining. In contrast, the removal of nucleotides from the 3′ terminus in a 5′-overhang scission site might not cause blunt-ends and the DNA strands might be still hold together if the gap is filled soon after. This model might explain the different effects of ara A on chromosome damage induced by *Pst* I from those induced by *Bam*H I.

The implication of the results obtained in the repair of DNA damage induced by ionizing radiation is of considerable importance. Compared to *Pvu* II and *Pst* I, the enhancement ratios by ara A treatment in γ-irradiated cells were reduced. This may imply that a mixture of types of DNA lesions, or dsb with different end-structures induced by irradiation and their sensitivity to ara A may vary. The possibility of facilitating the uptake of ara A in SLO treated cells and the combined effects of ara A with SLO on cells can not be excluded. The much greater enhancement ratio of ara A for *Pvu* II treatment in normal cells suggests that the majority of dsb induced by γ-rays are not blunt-ended. This has been suggested by Bryant (1989) and by the finding of Chung et al (1991) that the enhanced clastogenic effects of several

DNA repair inhibitors were considerably different between blunt-ended dsb caused by *Alu* I and DNA damage induced by irradiation.

In conclusion, RE-induced dsb are repaired not only by direct ligation but also by mechanisms involving DNA synthesis. The dsb caused by RE are probably subject to degradation at the broken end to a degree, depending somewhat on the end-structure of the lesion; blunt- and 3'-overhang cohesive dsb are more likely to be degraded than 5'-overhang cohesive dsb. This appears to be true for both AT and normal cell lines. The normal cell line (N-SW) responded more profoundly to ara A when compared to the AT cell lines (AT-KM and AT-PA). This may be an indication of an extensive degradation of dsb and a deficiency in the repair of the DNA damage induced in AT cells.

Chapter VI

Response of DNA Synthesis to RE Treatment in AT and Normal Cells

6. 1. Introduction

6. 2. Results

6. 2. 1. DNA synthesis in AT-PA and N-SW cells exposed to γ-irradiation

6. 2. 2. Effects of SLO on the incorporation of ^{3}H-TdR into DNA

6. 2. 3. DNA synthesis in *Pvu* II and *Eco*R I treated normal N-SW cells

6. 2. 4. DNA synthesis in AT-PA cells porated with high concentration of SLO

6. 2. 5. Effects of RE causing dsb with blunt- or cohesive-termini on DNA synthesis in N-SW cells

6. 2. 6. Cell cycle response after SLO poration and RE treatment

6. 3. Discussion

6. 1. Introduction

Inhibition of DNA synthesis is an intrinsic response of S-phase mammalian cells to ionizing radiation (Watanabe 1974, Painter and Young 1975, Makino and Okada 1975). It is believed that ionizing radiation leads to a blockage of the initiation of DNA replicons at low doses of radiation (Waters and Hildebrand 1975, Painter and Young 1976) and a termination of the chain elongation at higher doses (Watanabe 1974, Painter 1983, Mohamed et al 1986).

Besides ionizing radiation, a variety of DNA damaging agents are able to induce inhibition of DNA synthesis in mammalian cells. These include ultraviolet light at the wavelengths where either pyrimidine dimers or non-dimer DNA damage are induced (Kaufmann et al 1980, Painter 1985a, Rosenstein 1984), bleomycin and neocarzinostatin (Jaspers et al 1982, Shiloh and Becker 1982) both of which create strand breaks and alkali labile sites in DNA (Giloni et al 1981, Shiloh et al 1983a, Hatayama and Goldberg 1980), and an array of DNA alkylating agents, such as MNNG, MMS and EMS (Shiloh et al 1985, Lehmann 1982). Inhibition of DNA synthesis, therefore, seems to be a common response of normal cells to a broad spectrum of DNA lesions.

In contrast, radiosensitive AT cells are paradoxically characterised by an abnormal resistance of DNA synthesis after exposure to ionizing radiation (Houldsworth and Lavin 1980, Painter and Young 1980). The radioresistance of DNA synthesis in AT cells was suggested to be a result of completely resistant chain elongation and partially resistant replicon initiation (Painter 1985b, Mohamed et al 1986). Cellular radiosensitivity and radioresistance of DNA synthesis are thus two of the hallmarks of AT cells. The latter feature distinguishes AT from several other radiosensitive mammalian mutant lines, e.g., the Chinese hamster *xrs* lines, *irs* lines 1 and

3, murine L5178YS cells and *scid* cells (Jeggo 1985, Thacker and Ganesh 1990, Ockey 1983, Komutsu et al 1993), which all show normal radiation-induced inhibition of DNA synthesis. However, the Chinese hamster *irs*-2 line and V-group mutants, when compared with their wild-type parental (V79) line, show an AT-like resistant DNA synthesis to ionizing radiation (Jones et al 1990, Zdzienicka et al 1989).

Radioresistant DNA synthesis appears to be a dominant phenotype of AT cells. Cellular radiosensitivity in AT cells can be restored in AT x normal cell hybrids (Komatsu et al 1989) or in an AT cell line transfected with genomic DNA isolated from normal cells (Lehman et al 1986), whereas the reduced inhibition of DNA synthesis remained in the hybrids and the transfected AT cells. In the studies of Mohamed and Lavin (1986), the introduction of nuclear extracts from AT cells into normal cell lines caused an AT-like phenotype of radioresistent DNA synthesis.

The response of DNA replication of AT cells to other DNA damaging agents is quite contradictory. AT cells show a resistant inhibition of DNA synthesis after exposure to bleomycin or neocarzinostatin (Cramer and Painter 1981, Morris et al 1983, Jaspers et al 1982, Shiloh and Becker 1982, Cohen and Simpson 1983, Babilon et al 1985). The abnormal inhibition of DNA replication induced by bleomycin and neocarzinostatin is coupled to an enhanced cell killing in AT cells compared with normal cells (Taylor et al 1979, Shiloh et al 1982). After UV irradiation both DNA synthesis inhibition and cell viability are the same in AT and normal cells (Lehmann 1982, Jaspers et al 1982). AT cells show a normal inhibition of DNA synthesis (Jaspers et al 1982) but a cellular hypersensitivity to DNA alkylating agents (Hoar and Sargent 1976, Paterson and Smith 1979) although conflicting results for the cellular hypersensitivity of AT to the alkylating agents have been reported from other studies (Arlett et al 1982, Shiloh et al 1985). It is not yet clear which type of DNA damage is responsible for the hypersensitivity

of AT cells and what the correlation between reduced inhibition of DNA synthesis and decreased survival of AT cells after exposure to DNA damaging agents is.

In the previous chapters, it has been demonstrated that AT cells are more sensitive to RE-induced dsb than normal cells with respect to the induction of chromosomal aberrations. The results provide additional evidence to support the previous suggestion that AT cells are probably defective in dsb processing. A number of lines of evidence have demonstrated that RE-induced dsb mimic radiation effects (see Chapter 1), although there is no data on the effects of RE on DNA synthesis. Such questions arise as to whether RE-induced dsb lead to suppression of DNA synthesis, whether AT cells respond to RE-induced dsb differently from normal cells with respect to DNA synthesis, and whether RE-induced dsb with different end-structures are equally effective in causing inhibition of DNA synthesis. This chapter reports the investigations on the response of DNA synthesis to RE-induced dsb, which are either blunt- or cohesive-ended, in normal and AT lymphoblastoid cells.

6. 2. Results

6. 2. 1. DNA synthesis in AT-PA and N-SW cells exposed to γ-irradiation

The DNA synthesis capacity was examined in AT-PA and N-SW cells following γ-irradiation. 2 ml of cells (1×10^6/ml) were pulse-labelled for 30 min with ^{3}H-TdR (3.7×10^4 Bq/ml) 1 hour after exposure to γ-rays and the incorporation of ^{3}H-TdR into DNA was measured by the method as described in Chapter 2 section 2. 10. The results are shown in Figure 6. 1. As has expected, N-SW cells exhibited a dose-dependent suppression of DNA

synthesis, while the inhibition of DNA synthesis was markedly reduced in AT-PA cells.

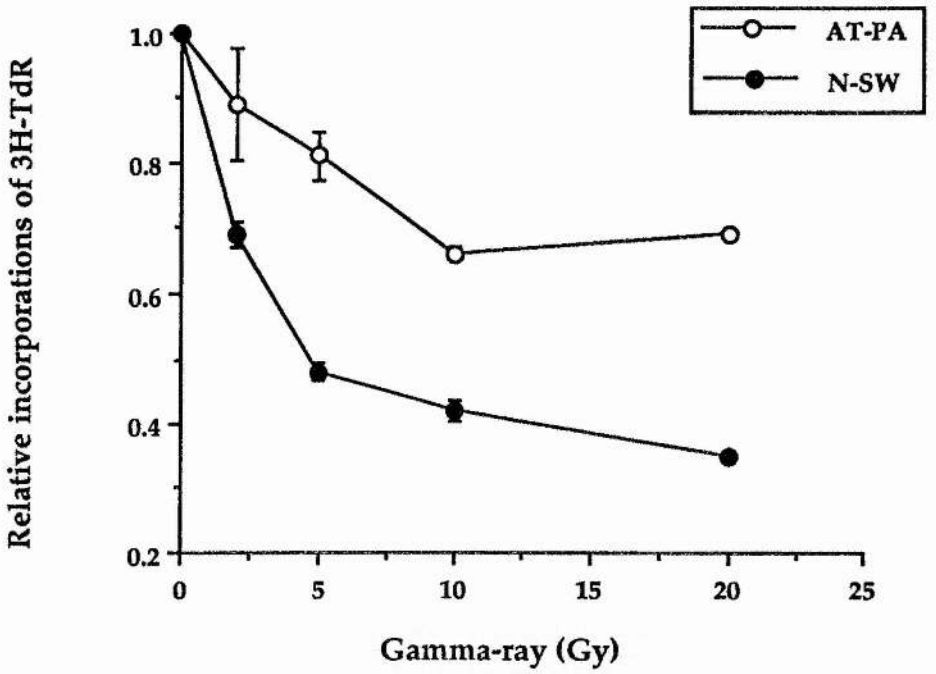

Figure 6. 1. Relative incorporation of ^{3}H-TdR (irradiated cells vs unirradiated cells) into DNA in AT-PA and N-SW cells as a function of γ-ray dose. Error bars represent standard errors of mean values of three independent experiments.

6. 2. 2. Effects of SLO on the incorporation of ^{3}H-TdR into DNA

SLO treatment was found to reduce the incorporation of ^{3}H-TdR into DNA in both AT and normal cells. When the cells were exposed to SLO at a concentration range of 0 - 0.16 units/ml for 5 min at room temperature followed by incubation with ^{3}H-TdR for an hour in the absence of SLO, the relative incorporation (SLO-treated versus untreated cells) of ^{3}H-TdR was observed to decrease with increasing SLO concentration in both AT and normal cells (Figure 6. 2).

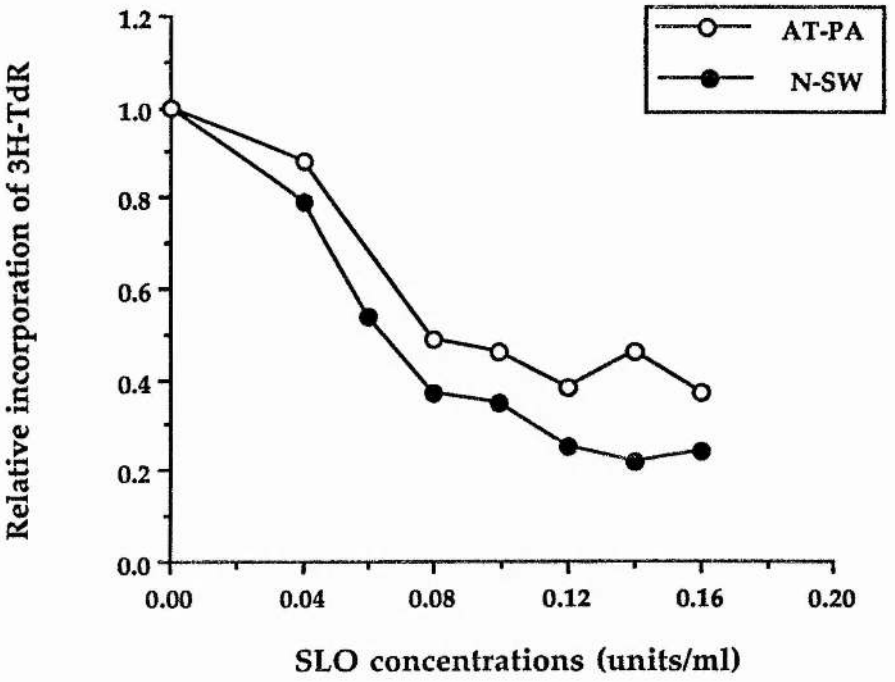

Figure 6. 2. Relative incorporation of ^{3}H-TdR (SLO-treated cells vs untreated cells) as a function of SLO concentration in AT-PA and N-SW cells. Cells were exposed to SLO for 5 min at room temperature and ^{3}H-labelled acid-insoluble material was measured as described in Chapter 2 (section 2. 10).

The SLO-induced inhibition of DNA synthesis was observed to be partially reversible during post-treatment incubation after poration of cells with SLO at 0.06 units/ml in both the cell lines. The relative incorporation of ^{3}H-TdR increased with increasing post-treatment incubation time as shown in Figure 6. 3. By 24 hours, the average incorporation in the cells treated with SLO alone reached approximately 70% and 80% of control level in AT-PA or N-SW cells, respectively.

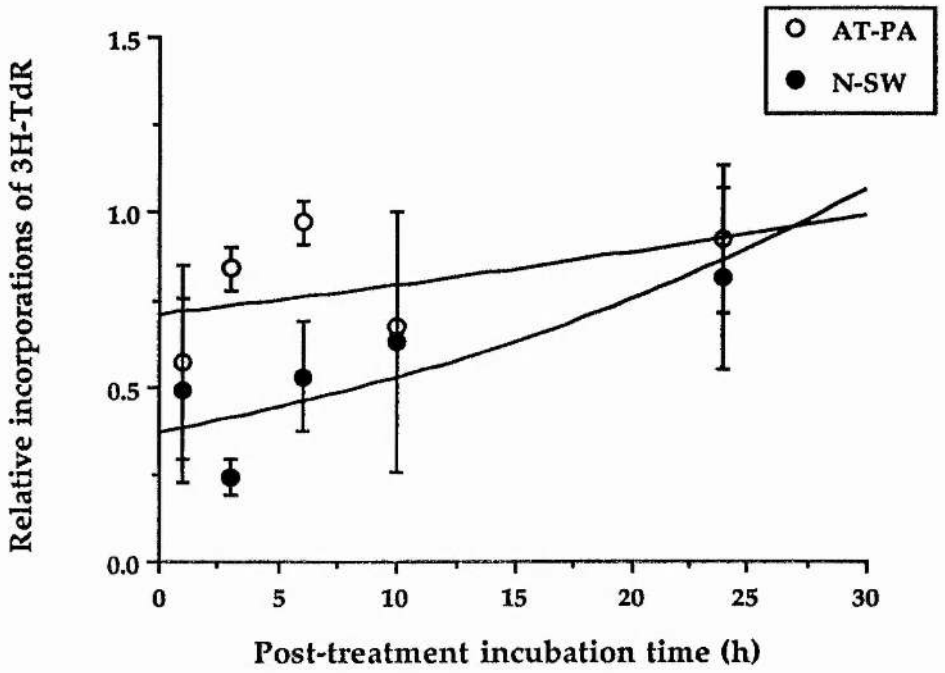

Figure 6. 3. Relative incorporation (SLO-treated cells vs untreated cells) of ^{3}H-TdR as a function of post-treatment incubation time. Error bars represent standard errors of mean values derived from 3 independent experiments.

6. 2. 3. DNA synthesis in *Pvu* II and *Eco*R I treated AT-PA and N-SW cells

Two restriction endonucleases, *Pvu* II and *Eco*R I, were used to examine the responses to dsb with blunt-ended (caused by *Pvu* II) or cohesive-ended (caused by *Eco*R I) in AT-PA and N-SW cells. The relative incorporation of ^{3}H-TdR was determined as a ratio of the incorporation in the cells treated with SLO and RE to those in the cells treated with SLO alone. The incorporation of ^{3}H-TdR was normalised to dpm per 10^{5} cells.

In N-SW cells, the rate of DNA synthesis was found to decrease with increasing incubation time following treatment with *Pvu* II (200 units/ml), while the rate of DNA synthesis remained at the control level after *Eco*R I treatment. The difference in the relative incorporation of ^{3}H-TdR between *Pvu* II and *Eco*R I treatment at 24 hours was significant ($p < 0.01$) in N-SW

cells. The decrease in DNA synthesis caused by *Pvu* II, however, was not observed in AT-PA cells, despite the presence of more chromosomal damage at this concentration at 24 hours in AT-PA cells when compared to N-SW cells as shown in Figure 3. 4 in Chapter 3. Figure 6. 4 also shows that *Eco*R I did not affect DNA synthesis in AT-PA cells.

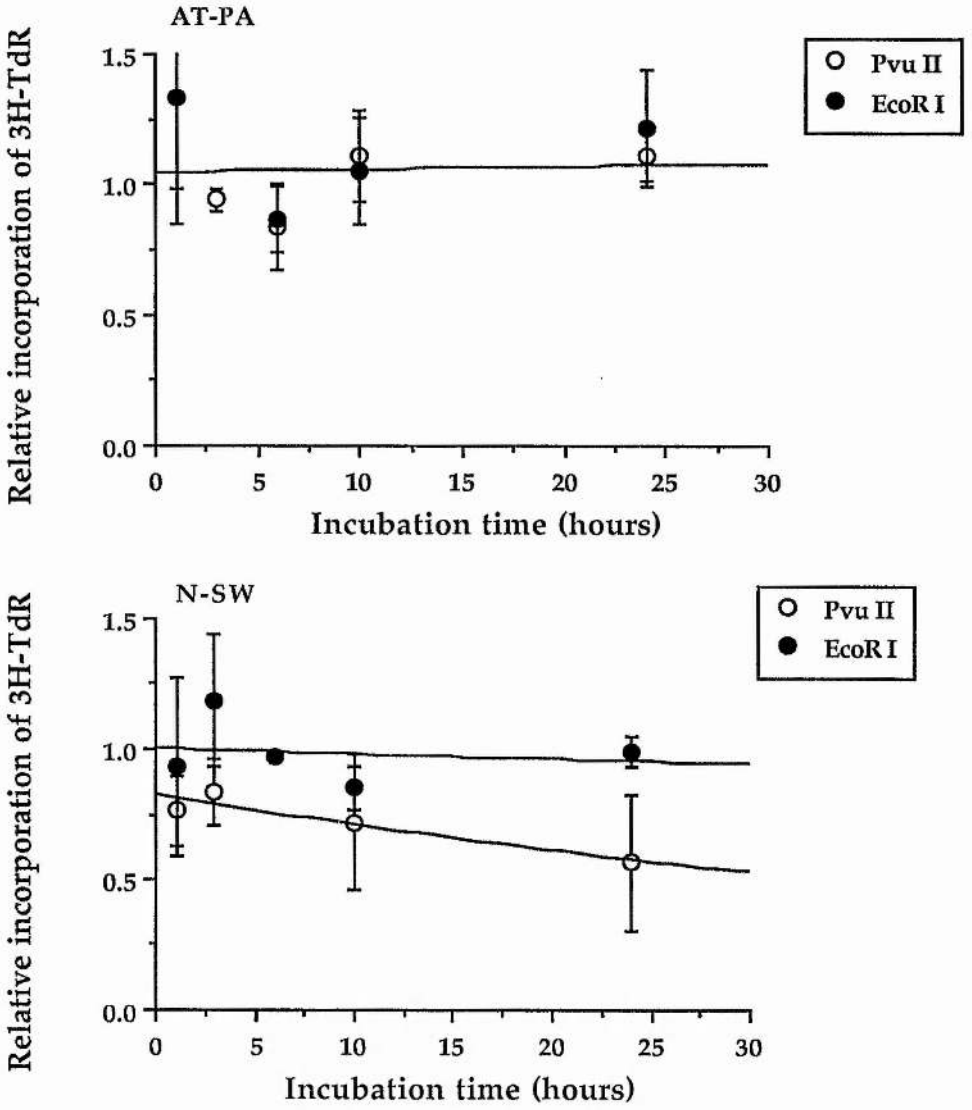

Figure 6. 4. Relative incorporation of ^{3}H-TdR as a function of post-treatment incubation for AT-PA and N-SW cells. Cells were porated with 0.06 units/ml SLO in the presence of 200 units/ml of *Pvu* II or *Eco*R I for 5 min. Mean values and standard errors of three experiments are presented.

6. 2. 4. DNA synthesis in AT-PA cells porated with high concentrations of SLO

AT-PA cells were found to be less sensitive to SLO treatment (Figure 4. 12 in Chapter 4) which may result in a reduced uptake of RE in these cells. This result could explain the reduction in the induction of dsb by *Pvu* II in AT cells compared with N-SW cells following 0.06 units/ml of SLO (Figure 4. 8). To compare the effects of *Pvu* II-induced dsb on the DNA synthesis between AT-PA and N-SW cell lines at the comparable level of dsb, DNA synthesis was measured in AT cells porated with 0.3 units/ml SLO, at which concentration the yield of dsb induced by *Pvu* II in AT-PA cells was found to be similar to that in N-SW porated with 0.06 units/ml of SLO (Figure 4. 9). The results are shown in Figure 6. 5. At 100 units/ml *Pvu* II, the incorporation of ^{3}H-TdR in AT cells was not significantly different following poration with 0.3 or 0.06 units/ml SLO ($p > 0.05$, student *t*-test), but was significantly lower than that in N-SW cells porated with 0.06 units/ml SLO ($p < 0.05$). In AT-PA cells treated with 200 units/ml *Pvu* II and porated with 0.3 units/ml of SLO, an increased inhibition of DNA synthesis was observed compared with those porated with the lower SLO concentration (0.06 units/ml) ($p < 0.05$), but was still reduced when compared with that in N-SW cells ($p = 0.06$). *Eco*R I treatment did not cause an inhibition of DNA synthesis in AT-PA cells even when porated with the higher concentration of SLO (Figure 6. 5).

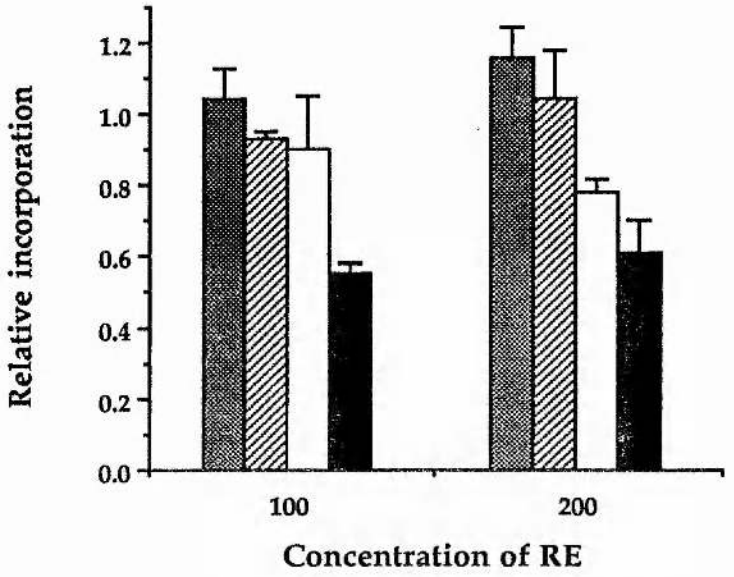

Figure 6. 5. Relative incorporation of ^{3}H-TdR 24 hours after treatment with *Eco*R I + SLO 0.3 units/ml (shaded); *Pvu* II + SLO 0.06 units/ml (hashed); *Pvu* II + SLO 0.3 units/ml (white) in AT-PA cells, and *Pvu* II + SLO 0.06 units/ml in N-SW cells (black). Error bars represent standard errors of mean values of 3 independent experiments.

6. 2. 5. Effects of RE causing dsb with blunt- or cohesive-termini on DNA synthesis in N-SW cells

Figure 6. 6 shows the dose-effect curves for *Pvu* II, *Eco*R V (both inducing blunt-ended dsb), and *Eco*R I and *Bam*H I (both inducing cohesive-ended dsb) in N-SW cells. A RE dose-dependent decline of DNA synthesis, which was steep initially and then levelled off, was observed following *Pvu* II and *Eco*R V treatment. Neither *Bam*H I nor *EcoR* I treatment caused a significant inhibition of DNA synthesis throughout the concentration range tested.

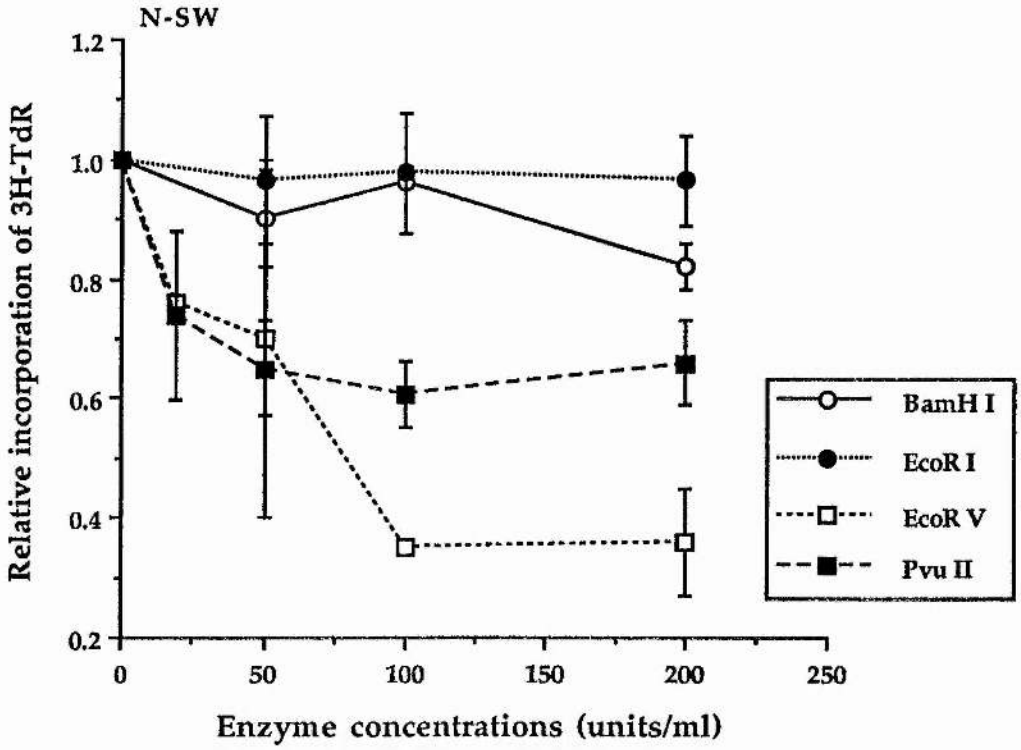

Figure 6. 6. Relative incorporation of ^{3}H-TdR in SLO (0.06 units/ml) porated N-SW cells 24 hours after treatment as a function of concentrations of *Pvu* II, *Eco*R V, *Bam*H I and *Eco*R I. Error bars represent standard errors of mean values of 4 (for *Eco*R I and *Pvu* II) and 2 (for *Eco*R V and *Bam*H I) independent experiments.

6. 2. 6. Cell cycle response after SLO poration and RE treatment

To compare the responses of DNA synthesis and of cell cycle in cells after SLO and RE treatment, AT-PA and N-SW cells were exposed to 0.06 units/ml of SLO alone or combined with *Pvu* II (100 units/ml), and the percentage of cells at G_1, S or G_2/M phases was determined by a flow cytometric technique (Chapter 2 section 2. 11) at various post-treatment time intervals. The results show that AT-PA cells were not significantly changed in the progression of cell cycle in any phase by the treatment with SLO alone or combined with *Pvu* II (Figure 6. 7) when compared with untreated control cells. The combination treatment with SLO and *Pvu* II,

however, exerted effects on cell cycle progression of N-SW cells (Figure 6. 8). At 4 hours post-treatment, the cell population in S phase treated with SLO and *Pvu* II increased significantly ($p < 0.05$) compared to the control and those treated with SLO alone. The difference in the number of S phase cells between treatment with and without *Pvu* II diminished after 24 hours incubation, while a difference between the G_2/M phase population after *Pvu* II treatment and control cells became significant ($p < 0.05$). These results indicate that *Pvu* II treatment influenced the cell cycle progression in normal but not in AT cells.

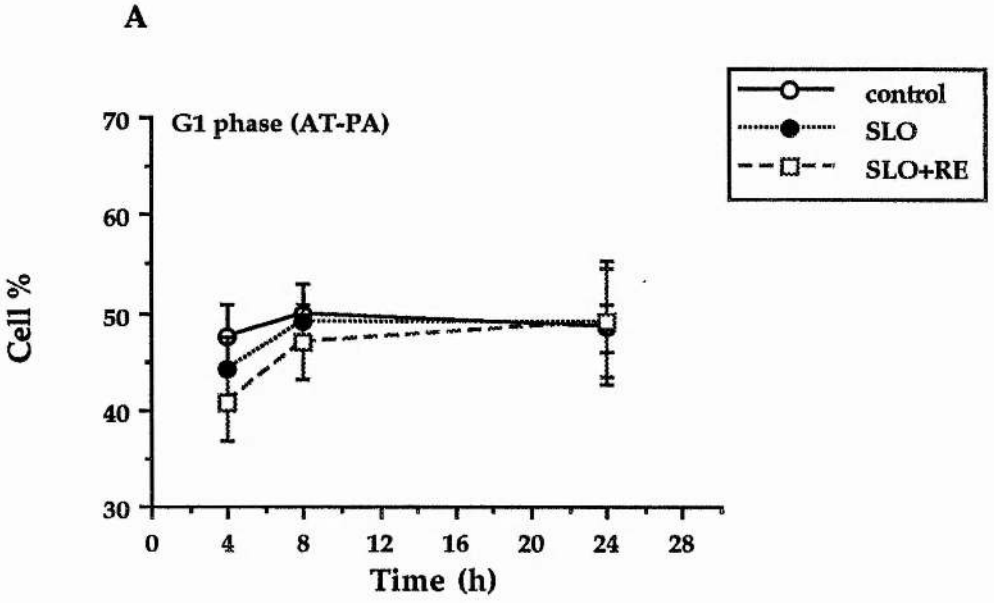

Figure 6. 7 (A). AT-PA cell population in G_1 phase of cell cycle after SLO (0.06 units/ml) or SLO+*Pvu* II (100 units/ml) treatment. Error bars represent standard errors of mean values obtained from 2 - 3 independent experiments.

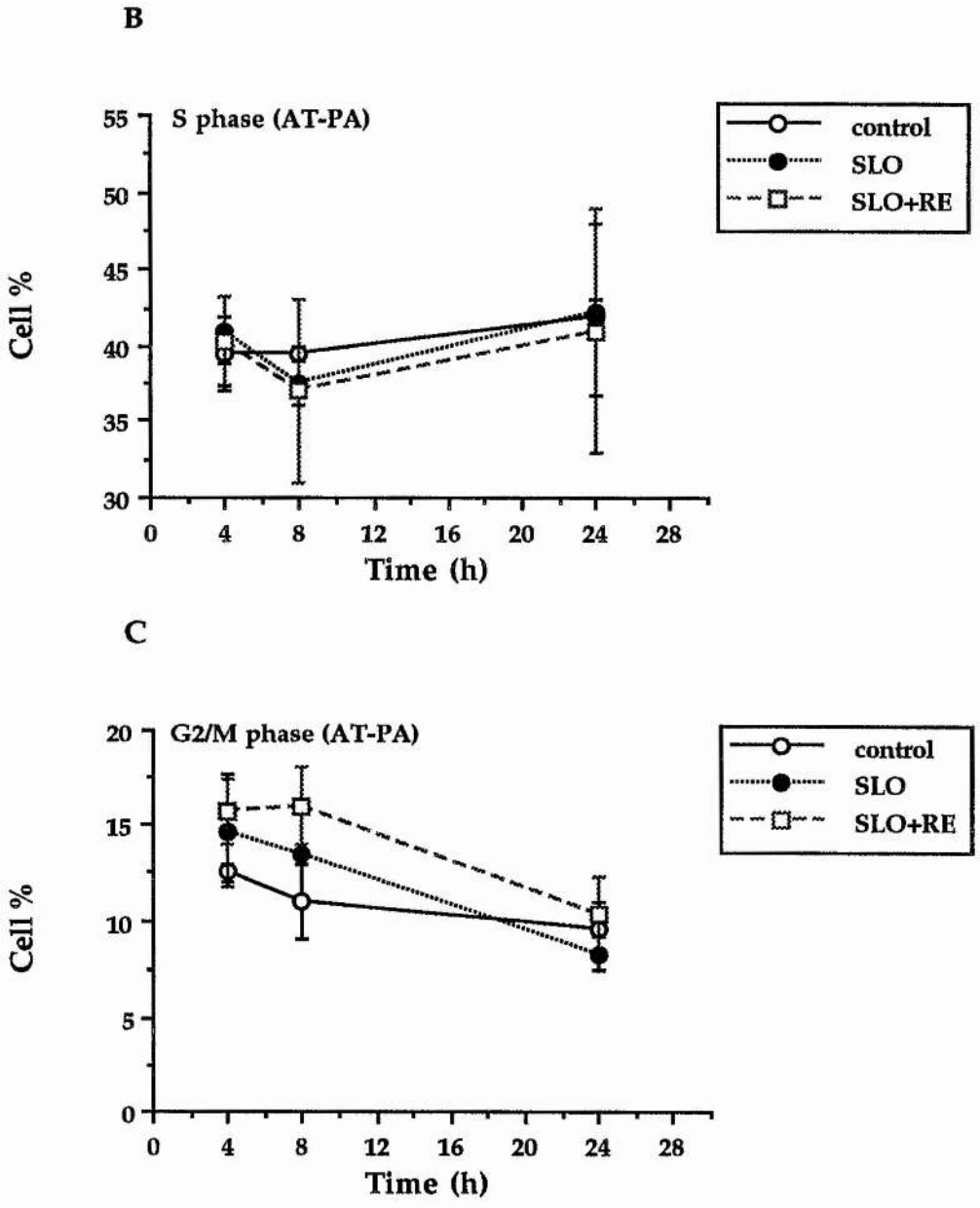

Figure 6. 7 (continue). AT-PA cell population in S (panel B) and G_2/M (panel C) phases of cell cycle after SLO (0.06 units/ml) or SLO+*Pvu* II (100 units/ml) treatment. Error bars represent standard errors of mean values obtained from 2 - 3 independent experiments.

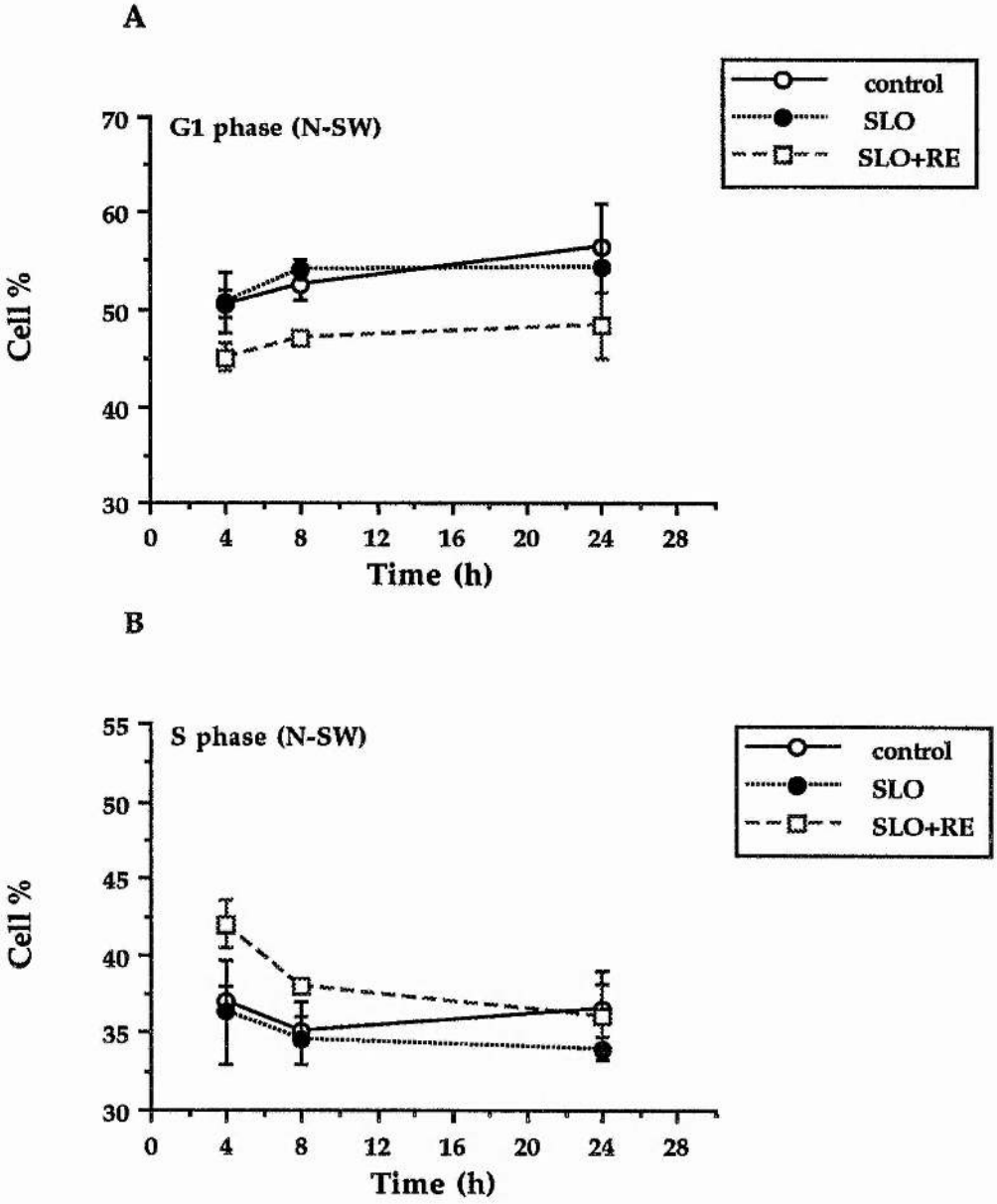

Figure 6. 8. N-SW cell population in G_1 (panel A) and S (panel B) phases of cell cycle after SLO (0.06 units/ml) or SLO+*Pvu* II (100 units/ml) treatment. Error bars represent standard errors of mean values obtained from 2 - 3 independent experiments.

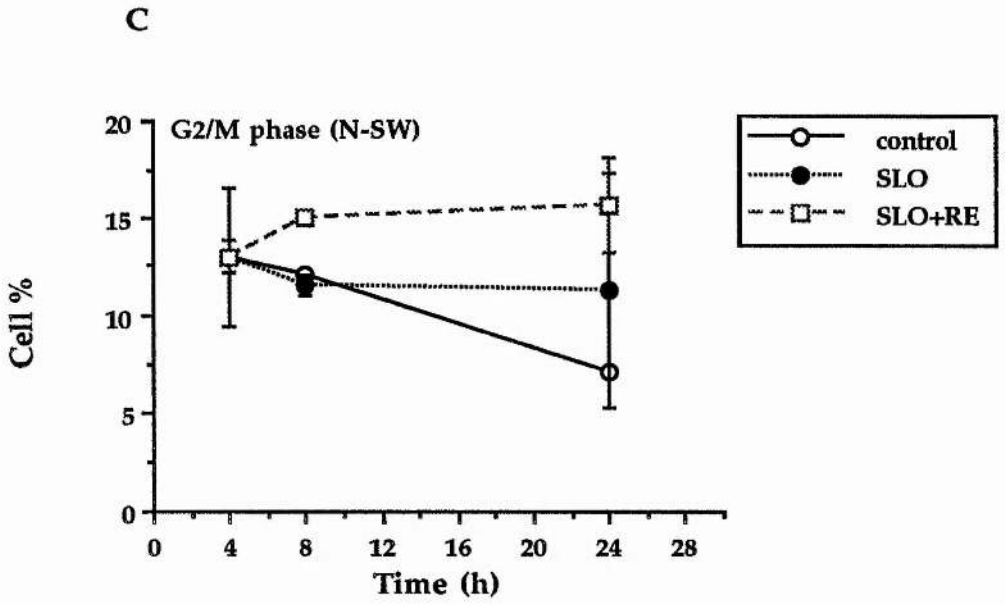

Figure 6. 8 (continue). N-SW cell population in G_2/M (panel C) phase of cell cycle after SLO (0.06 units/ml) or SLO+*Pvu* II (100 units/ml) treatment. Error bars represent standard errors of mean values obtained from 2 - 3 independent experiments.

6. 3. Discussion

The inhibition of DNA synthesis was demonstrated in normal (N-SW) cells treated with *Pvu* II and *Eco*R V. Both enzymes cause dsb with blunt-termini. In contrast, *EcoR* I and *Bam*H I, which generate cohesive-ended dsb in DNA, exhibited a minor effect on the rate of DNA synthesis in N-SW cells. The diminished inhibition of DNA synthesis in the cells containing cohesive-ended dsb correlates with a reduced clonogenic and clastogenic effectiveness associated with cohesive-ended dsb when compared with cells containing blunt-ended dsb (see review Bryant 1989 and Chapter 4 and 5).

Although both blunt- and cohesive-ended dsb have clean 3'-hydroxyl and 5'-phosphoryl termini, the repair of these two types of dsb seems to be quite different. Costa and Bryant (1991a) have shown that blunt-ended dsb

induced by *Pvu* II accumulate in CHO cells with increasing incubation time, while cohesive-ended dsb induced by *Bam*H I did not. The reduced accumulation of cohesive-ended dsb in CHO cells is thought to be due to the fast rejoining of this type of damage (Costa and Bryant 1991a). This implies that the presence and accumulation of DNA breaks is necessary for the induction of the inhibition of DNA synthesis.

A biphasic inhibition of DNA synthesis was found in N-SW cells exposed to γ-irradiation (Figure 6. 1). The "steep" part of the dose-effect curve at low doses (< 5Gy) of radiation was suggested to be a result of inhibition of DNA replicon initiation, whereas the less steep decline at high doses of radiation was proposed to be a result of termination of chain elongation (Painter 1985b). It has been proposed that inhibition of initiation of DNA synthesis in replicons is mediated by a *trans*-acting factor which is induced by DNA damage *per se*, and which inhibits DNA replication in a large chromatin domain (Lamb et al 1989). The block of chain elongation is presumably due to existence of damage(s) in that replicon (Povirk and Painter 1976, Painter 1985b). After *Pvu* II treatment, a steep curve associated with the inhibition of DNA synthesis was observed in normal cells at lower concentrations of the enzyme, but there was a lack of further inhibition at higher concentrations (Figure 6. 4). This might be interpreted by considering the cutting frequencies of RE (theoretically 1 cut every 3000 bp for *Pvu* II) that RE-induced dsb are not abundant enough to induce a noticeable block of chain elongation in duplicating replicons, but is sufficient to signal chromatin to down-regulate replicon initiation.

AT lymphoblastoid cells exhibited a reduced inhibition of DNA synthesis after *Pvu* II treatment compared to N-SW cells. Since different efficiencies of poration of SLO were measured in AT-PA and N-SW lymphoblastoid cell lines (Chapter 4), the comparison of the DNA synthesis rates between AT-PA and N-SW cell lines was made under the poration

conditions where either a clastogenic hyper-sensitivity to *Pvu* II was seen in the AT cells (0.06 units/ml of SLO for both cell lines), or where the number of dsb induced by *Pvu* II was comparable in both cell lines (0.3 units/ml of SLO for AT-PA cells and 0.06 units/ml of SLO for N-SW cells). Although an increased number of dsb and a decreased incorporation of ^{3}H-TdR induced by *Pvu* II was found in AT cells porated with 0.3 units/ml of SLO, compared to those in the cells porated with 0.06 units/ml of SLO, the overall rates of DNA synthesis in AT cells treated with either concentration of SLO were still higher than the normal cells.

Following *Pvu* II treatment of SLO porated cells, an increased population of cells in S phase was observed in the N-SW cell line 4 hours post-treatment incubation, but not after 24 hours (Figure 6. 8). This implies that cells were delayed during DNA replication and were thus temporarily arrested in S phase. A G_2 delay was also observed in N-SW cells 24 hours post-treatment (Figure 6. 8). In contrast, the perturbation of cell cycle induced by *Pvu* II was not obvious in AT cells (Figure 6. 7).

Like other DNA damaging agents which disrupt the integrity of DNA structure, e.g., ionizing radiation, bleomycin or neocarzinostatin, RE induce an abnormal response of DNA synthesis in AT cells which is accompanied by both chromosomal and cellular hypersensitivity (Chapter 4 and Liu and Bryant 1993, Costa and Thacker 1993). These results suggest the presence of a defect in responding to RE-induced dsb may exist in AT cells which may involve damage recognition and the regulation of cell cycle progression.

In conclusion, RE-induced blunt-ended dsb can cause an inhibition of DNA synthesis in normal lymphoblastoid cells porated with SLO, which indicates that this type of dsb may be one of the main lesions responsible for the reduction of the rate of DNA synthesis observed after ionizing radiation. Most interestingly, the AT cell line (AT-PA) shows a reduced inhibition of

DNA synthesis in response to RE-induced dsb, similar to that observed after exposure to ionizing radiation.

Chapter VII

Introduction of Normal Cell Protein Extracts Into AT Cells -- An Attempt to Restore the Chromosomal Sensitivity of AT to γ-rays or Restriction Enzyme

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7. 3. 7. DNA-protein binding property in AT-PA and N-SW cell extracts

7. 4. Discussion

7. 1. Introduction

The miscellaneous anomalies of AT individuals, including hypersensitivity to DNA damage, are thought to be a result of alteration in the function of AT gene(s), a gene which has been localised to human chromosome 11q22-23 for all 4 complementation groups (A, C, D and E) identified so far (Gatti et al, 1988, Ziv et al 1991, Lambert et al 1991, Taylor et al 1994). The AT gene for complementation group D (ATDC gene) has been shown to be present as a single copy in the human genome (Kapp et al 1992). Neither a large rearrangement in the ATDC gene sequence nor abnormal RNA expression is evident in AT5BIVA cells, indicating that a point mutation or a small rearrangement may be involved (Kapp et al 1992). The biochemical properties and functions of AT gene products are currently unknown.

Evidence has indicated that the products of normal (repair-efficient) human gene(s) that complement the AT gene, are able to confer normal radiosensitivity to AT cells when these normal gene(s) are expressed in AT cells. By the transfer of genomic DNA derived from normal cells into AT cells, the sensitivity of AT cells to ionizing radiation was restored as assayed by colony-formation (Lehmann et al 1986). Using the microcell-mediated chromosome transfer technique, human normal chromosome 11 was transferred into AT cells and was shown to confer normal cellular (Lambert et al 1991) and chromosomal sensitivity (Kodama et al 1992) in AT cells in response to DNA damaging agents. It is not known so far whether the protein factor(s) encoded for by genes on chromosome 11 are involved in determining chromatin structure and/or in the processing of DNA damage.

A cell-free system for the repair of dsb induced by RE in human nuclear extract has been described and has proved useful for the investigation of defects in DNA damage repair in AT cells (North et al 1990, Thacker et al 1993, Ganesh et al 1993). Observations from these studies have

shown that RE-induced breaks in plasmid DNA could be correctly rejoined by human nuclear extract although a proportion of the molecules were misrejoined. Extracts derived from AT cells resulted in an elevated frequency of misrejoining relative to normal cell extracts. The rejoining fidelity was found to be 10 fold lower in AT nuclear extracts than in normal extracts, although the rejoining efficiency was observed to be normal (North et al 1990) as in where AT cell (Cox et al 1984). The extract-mediated misrepair involved complex events, such as deletion and insertion in addition to point mutation (Thacker et al 1993). The pattern of mutation in the recircularized plasmids treated with nuclear extract *in vitro* was found to be the same as that in transfection experiments where RE-cut vectors were processed by cells (Rünger et al 1992, Powell et al 1993). Analysis of misrejoined molecules show that deletion and insertion occurred predominantly between short direct repeats (2 - 6 bp) at the scission sites, a phenomenon which was also found in γ-irradiated shuttle vectors after being processed by transfected cells (Sikpi et al 1992). These findings indicated that the extract contain some protein(s) or enzyme(s) which are possibly involved in the processing of DNA damage. Evidence that normal but not AT nuclear extract offer a proficient DNA repair suggests the possibility of the restoration of DNA repair capacity in AT cells by normal extract proteins.

Evidence for the existence of proteins that cause the aberrant response of AT cells to ionizing radiation is also obtained from AT cell extract. Introduction of cell extracts derived from 4 AT cell lines into 2 irradiated normal cell lines led to an increase in the capacity for DNA synthesis in normal cells following radiation (Mohamed and Lavin 1986). This result was consistent with observations that radioresistant DNA synthesis remained in AT x normal hybrid cells (Kodama et al 1989), in AT cells transfected with normal genomic DNA (Lehmann et al 1986) and in AT

cells containing a normal chromosome 11 (Lambert et al 1991), although all of those means led to a restoration of cellular or chromosomal sensitivity to ionizing radiation in AT cells. The factor(s) responsible for the abnormal response of DNA replication in irradiated AT cells therefore seem to be extractable and transportable.

A similar attempt to introduce extracts from normal human cells into AT-PA cells to restore the increased chromosomal sensitivity to RE- or γ-ray induced damage is described in this chapter. The utilisation of SLO has ensured the efficient introduction of RE into cells to cause chromosomal aberrations (Chapter 4). This approach was used in the present study to porate normal human (N-SW) cell extracts into AT-PA cells and to investigate the resultant frequencies of chromosomal aberrations induced by ionizing radiation or RE.

7. 2. Materials and Methods

7. 2. 1. Cell lines

Two cell lines, AT-PA and N-SW were used for the experiments described in this chapter. Both cell lines were maintained in suspension culture in RPMI/FCS medium as described in section 2. 1 (Chapter 2).

7. 2. 2. Preparation of nuclear and cell extracts

Nuclear extract was prepared from log-phase AT-PA or N-SW cell lines using the method of North et al (1990) with some modifications. Cell extract was prepared from log-phase N-SW cells using the method described by Mohamed and Lavin (1986). The details of extraction of nuclear and

cellular extracts are described in section 2. 12 (Chapter 2). The extracts were aliquoted and stored at -20 °C before use.

7. 2. 3. Fractionation of cell extract by phospho-cellulose chromatography

All the procedures were performed at 0 - 4 °C and proteins monitored using a UV monitor (LKB Bromma). The cell extract was diluted with 50 mmol/l phosphate buffer (KPO_4, pH 7.5) to 0.1 - 0.3 mg/ml, and loaded on to a 1 x 8 cm P11 phospho-cellulose (Whatman) column, pre-equilibrated with 50 mmol/l KPO_4. Unbound proteins were washed from the column with 50 mmol/l KPO_4. Proteins remaining on the column were eluted stepwise with KPO_4 at required concentrations as indicated in Table 7. 1, containing 1 mmol/l DTT and 0.6 mmol/l EDTA. The eluates were precipitated by 70% ammonium sulphate. The resulting protein pellets were dissolved in 1 ml of extraction buffer (containing Hepes 40 mmol/l, pH 7.0; KCl 80 mmol/l; DTT 1 mmol/l; EGTA 1 mmol/l; $MgCl_2$ 4 mmol/l and ATP 2 mmol/l) and applied to an Amicon 10 ultrafilter for desalination by centrifugation at 7,700 x g for 2 hours and washed once with 2 ml of the extraction buffer. The residual protein solution above the filter was collected and stored at -20 °C. The concentration of protein was measured using the Bio-Rad assay (Chapter 2 section 2. 12). The yield of protein in each fraction is shown in Table 7. 1.

Table 7. 1. The yields of extract proteins in the fractions.

Fraction	KPO_4 buffer (mmol/l)	Protein concentration (µg/µl)	Total volume (µl)	Total protein (µg)
Exp 1				
F_{50}	50	6.09	245	1492
F_{125}	125	0.25	90	22.5
F_{200}	200	0.15	300	44.4
F_{300}	300	0.05	90	4.7
Exp. 2				
F_{50}	50	9.8	244	2391
F_{75}	75	0.2 - 0.5[a]	130	26 - 65[b]
F_{100}	100	0.2 - 0.5[a]	120	24 - 60[b]
F_{125}	125	0.05 - 0.1[a]	140	7 - 14[b]
F_{200}	200	0.22	330	72.6
F_{300}	300	UD	77	-

[a]: Protein concentrations were estimated from the absorbance 280 nm profile.
[b]: Estimated amount of protein.
UD: Protein concentration was undetectable.

7. 2. 4. Introduction of cell extracts into cells

The nuclear or whole cell extracts were introduced into living cells by SLO poration. In the experiments with whole cell extract, the cell pellet was mixed with the extract in the presence of SLO (approximately 0.08 units/ml)

in a total volume of approximately 70 µl. Typically, 1 x 10^6 log-phase cells were centrifuged in 1.5 ml Eppendorf tube and the cell pellet was resuspended in 50 µl of the extract or the extraction buffer. 3.2 µl of SLO (stock solution: 1.9 units/ml) was added to the cell suspension and the exposure was carried out for 10 min at room temperature. Following exposure, the cell suspension was diluted by the addition of 1 ml of RPMI/FCS medium followed by immediate centrifugation to remove SLO. The supernatant was aspirated and the cells were resuspended in 1 ml RPMI/FCS medium. Irradiation with 0.3 Gy γ-rays was carried out immediately after poration. Cells were incubated at 37 °C for 1 hour followed by incubation with colcemid (0.04 µg/ml) for a further 3 hours before fixation for subsequent chromosome analysis.

An alternative approach was used for the introduction of nuclear extract into AT-PA cells. Cells (1 x 10^6) suspended in HBSS/BSA were mixed with 100 µg of nuclear proteins, 0.3 units/ml of SLO and 125 units/ml of *Pvu* II in a total volume of 400 µl for 5 min. *Pvu* II was previously purified by the method described in section 2. 2. The removal of SLO was performed as previously described. The cells were incubated for 1 hour then incubated with colcemid (0.04 µg/ml) for a further 4 hours before fixation.

For γ-irradiation, the cells were exposed to 0.3 units/ml of SLO and 100 µg/ml of nuclear proteins (as described above) in the absence of *Pvu* II. Following treatment, cells were immediately irradiated with 0.3 Gy γ-rays and post-irradiation incubated for 4 hours (3 hours with colcemid).

7. 2. 5. Linearization of plasmid DNA with restriction endonucleases

Plasmid pBR322 was digested with *Pvu* II or *Eco*R I in the required reaction buffer at 37 °C for 2 hours. After the reaction, the mixture was diluted with distilled H_2O to a volume of over 50 µl and an equal volume

of phenol-chloroform-isoamylalcohol (24 : 24 : 1 v/v/v) was added for the extraction of plasmid DNA. Following ethanol precipitation, the plasmid DNA was redissolved in TE (Tris-HCl 10 mmol/l, pH 8.0; EDTA 1 mmol/l) at a concentration of 0.2 μg/μl. The linearized plasmid was assayed by agarose gel electrophoresis. The gels were run in the presence of ethidium bromide (EB) unless otherwise indicated.

7. 2. 6. *In vitro* assay of enzyme activities in the extracts

Assay of DNase activity

The contamination by DNases was assayed in the nuclear and cellular extracts. For each fraction of the cellular extract, 10 μl was mixed with 1 μl of circular or linear pBR322 (0.2 - 0.3 μg/μl) and incubated at 37 °C for 4 hours. For nuclear extract, 1 μl of pBR322 was mixed with 2 μl of nuclear extract in a reaction buffer containing 40 mmol/l of Tris-HCl, pH 7.5, 6 mmol/l of $MgCl_2$ in total volume of 10 μl. The reactions were carried out for 2 hours or overnight. To terminate the reaction, 2 μl of 10% SDS was added and the samples were subjected to electrophoresis on an agarose gel (0.8 %) in TBE buffer at a constant voltage (30 V) overnight.

Assay of DNA break rejoining activity

The rejoining of restriction enzyme-linearized pBR322 by the nuclear extract was examined. The reaction mixture (10 μl) contained 60 mmol/l Tris-HCl, pH 7.8; 10 mmol/l $MgCl_2$; 10 mmol/l 2-mercaptoethanol; 1 mmol/l ATP, 10% glycerol, 0.35 μg linear pBR322, and nuclear extract or T_4 ligase (Gibco-BRL) as required. Reactions were carried out at room temperature (approximately 20 °C) or 37 °C as required, and terminated by the addition of 2 μl SDS (final concentration: 1% w/v) or placing at -20 °C. Samples were electrophoresed as described above.

Assay of topoisomerase activity

Supercoiled pBR322 (0.35 µg) was incubated with the extract at 37 °C for 4 hours in a total volume of 10 µl and the relaxation of supercoiled plasmid by the extract was tested. Experiments with cell extract was performed in a buffer containing 40 mmol/l Hepes, pH 7.0; 80 mmol/l KCl; 1 mmol/l DTT; 1 mmol/l EGTA and 4 mmol/l $MgCl_2$, supplemented with or without 2 mmol/l ATP, while with nuclear extract, the reaction was carried out in a buffer containing 20 mmol/l Tris-HCl, pH 7.5; 0.5 mmol/l $MgCl_2$; 2 mmol/l $CaCl_2$ and 0.5 mmol/l KCl. The samples were run on an agarose gel without EB after addition of SDS as described above.

7. 2. 7. Random primer labelling of DNA

Sonicated and single stranded calf thymus DNA (Sigma) 25 ng were labelled with α-^{32}P-dCTP (Amersham) at a concentration of 3.7×10^7 Bq/ml (1 µCi/µl), by using a random primer labelling kit (Ready To Go™ DNA labelling Kit, purchased from Pharmacia) in a total volume of 50 µl for 1 hour at room temperature. The reaction mixture was subsequently loaded onto a Sephadex G50 column (Nick™Spin columns, Pharmacia) pre-equilibrated with TE buffer and centrifuged at 400 rpm for 4 min to separate labelled DNA from free ^{32}P-dCTP. Labelled DNA was eluted and collected, while free ^{32}P-dCTP retained in the column.

7. 2. 8. Assay of DNA-protein binding activity in cell extracts

A nitrocellulose filter binding assay was used for determination of DNA-protein binding activity in cell extracts. Cell extract proteins (3 µg) were mixed with 0.2 ng of ^{32}P-labelled DNA (approximately 5×10^5 - 5×10^6

cpm) and appropriate amounts (0 - 10 µg) of sonicated unlabeled (cold) natural calf thymus DNA (Sigma) in a buffer containing 25 mmol/l of Tris-HCl, pH 7.5, 10 mmol/l of EDTA, 10 mmol/l of 2-merchaptoethanol, 0.5 mmol/l of KCl and 0.5 mmol/l of $MgCl_2$, in a total volume of 30 µl. Addition of 3 µg of BSA or histone proteins (histones H1 and H2a, Sigma) instead of cell extract was utilized as background or positive control respectively. Reaction mixtures were incubated at 37 °C for 90 min and afterwards loaded onto nitrocellulose filters (pore size: 0.45 µm, Whatman). Filters were washed 4 times with 2 ml ice cold buffer containing 25 mmol/l Tris-HCl, pH 7.5, 10 mmol/l EDTA, 0.5 mmol/l KCl and 0.5 mmol/l $MgCl_2$. Radioactivity on the filter was counted in 4 ml Filter-Count™ (Packard) in a scintilation counter.

DNA binding activity of cell extract protein was calculated by the following formula:

$$\text{DNA-protein binding activity} = [(cpm_x - cpm_b) / cpm_t] \times (W_{cold} + W_{labelled})$$

where cpm_x: cpm for sample; cpm_b: cpm for background (BSA); cpm_t: cpm for total ^{32}P-labelled DNA added; W_{cold}: µg of cold DNA; $W_{labelled}$: µg of labelled DNA.

7. 3. Results

7. 3. 1. *In vitro* assays of DNA rejoining, DNases and topoisomerases activity in nuclear extract

Approximately 2 - 4 x 10^8 cells of each cell line were used for the preparation of nuclear extract. The yield was approximately 90 - 100 µg

nuclear proteins per 10^8 cells. The protein concentrations in nuclear extract of both AT-PA and N-SW cell lines were approximately 1 μg/μl.

The DNA rejoining activity of nuclear extract was measured using *Pvu* II- or *Eco*R I-linearized pBR322. The results are shown in Figure 7. 1. The reactions were carried out in a T_4 ligase buffer (Figure 7. 1A) or in an otherwise buffered condition (see 7. 2. 5) similar to that used by North et al (1990) (Figure 7. 1B). Neither N-SW nor AT-PA nuclear extract demonstrated break rejoining activities under these testing conditions, although efficient ligation by T_4 ligase was observed.

The activity of topoisomerase (Topo I) in relaxation of supercoiled pBR322 was observed in N-SW as well as in AT-PA nuclear extract at either 37 °C or 4 °C (Figure 7. 2). When circular or linearized pBR322 were incubated with nuclear extract at 37 °C for up to 18 hours, no obvious digestion of DNA was observed (Figure 7. 3), indicating that there was no detectable DNase activity in the nuclear extract.

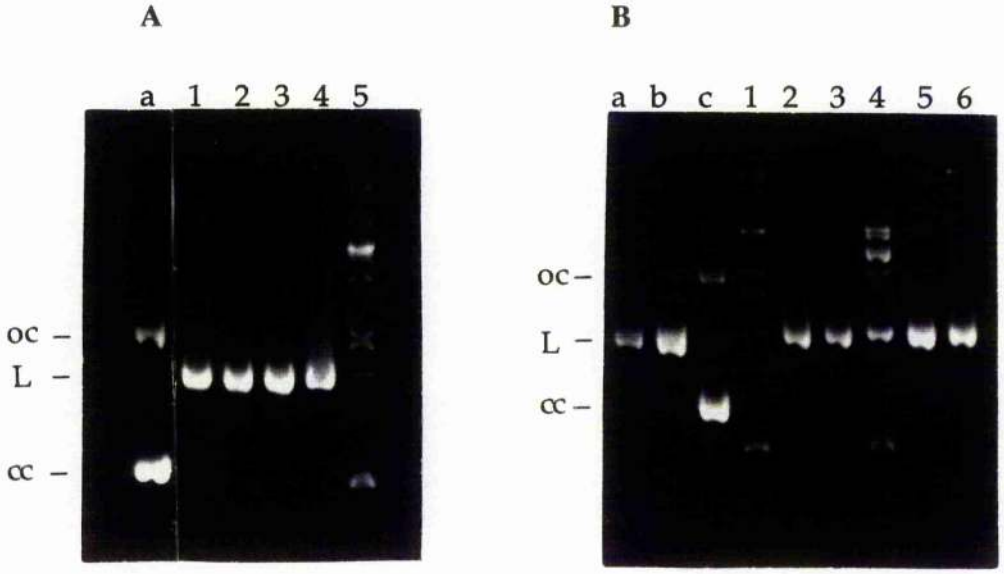

Figure 7. 1. Effects of nuclear extracts on DNA break rejoining. (A) Reactions in T_4 ligase buffer at room temperature for 16 hours. Lane a: pBR322 0.35μg. Lanes 1 - 5: pBR322/*Eco*R I 0.2 μg incubated without (lane 1) or with N-SW extract 1 μg (lane 2); 5 μg (lane 3) and 10 μg (lane 4). Lane 5: T_4 ligase 1 unit. (B) Reactions in buffer (see 7. 2. 6) at room temperature for 20 hours. Lane a: pBR322/*Eco*R I 0.2 μg; lane b: pBR322/*Pvu* II 0.3 μg; lane c: pBR322 0.35 μg; lanes 1 - 3: pBR322/*Eco*R I with T_4 ligase 1 unit (lane 1), with 2 μg AT-PA extract (lane 2) or with 2 μg N-SW extract (lane 3). Lanes 4 - 6: pBR322/*Pvu* II with T_4 ligase 1 unit (lane 4), 1.7 μg AT-PA extract (lane 5) or 2 μg N-SW extract (lane 6). cc: open circles; L: linear; oc: open circles.

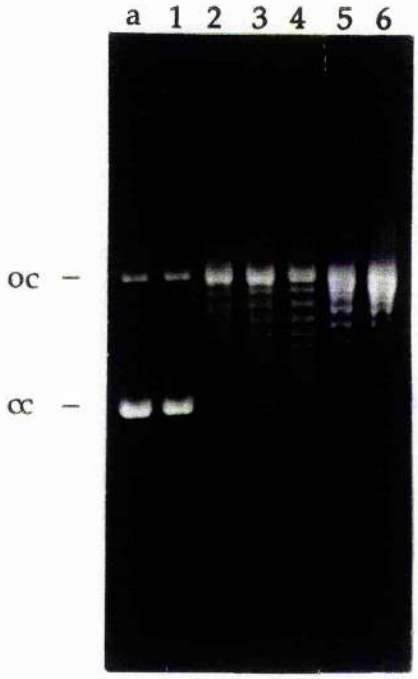

Figure 7. 2. Effects of nuclear extracts on the relaxation of supercoiled pBR322. 2 μg of nuclear extract from N-SW cells were used. Lane a: pBR322. Reactions were carried out at 37 °C (lanes 1 - 3) in buffer for 4 h (lane 1), in N-SW extract for 4 h (lane 2) and in N-SW extract for 2 h (lane 3), or at 4 °C (lanes 4 - 6) in N-SW extract for 4 h (lanes 4 and 6, separate preparations) and in AT-PA extract (1.7 μg) for 4 h (lane 5). Gels were run in the absence of EB and stained with EB following electrophoresis. cc: open circles; oc: open circles.

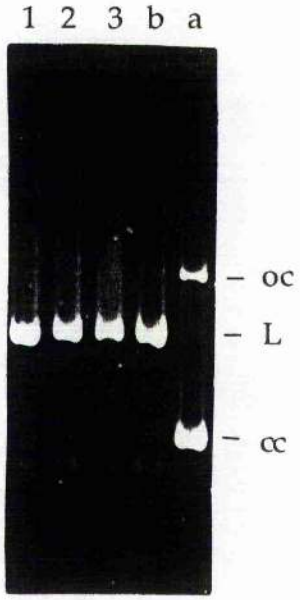

Figure 7. 3. Effects of nuclear extracts (2 μg) on DNA digestion at 37 °C for 18 hours. Lane a: pBR322 0.35 μg; lane b: linear pBR322 0.3 μg. Lanes 1 - 3: linear pBR322 in buffer C (lane 1), in AT-PA extract (lane 2) and in N-SW extract (lane 3). cc: open circles; L: linear; oc: open circles.

7. 3. 2. Reduction in frequencies of chromosomal aberrations induced by *Pvu* II in AT-PA cells by introduction of normal nuclear extract

The nuclear extract from N-SW or AT-PA cells was introduced into AT-PA cells by SLO poration at 0.3 units/ml and the restoration of normal frequencies of chromosomal aberrations induced by *Pvu* II or γ-rays in AT-PA cells were investigated. The use of a high concentration of SLO (0.3 units/ml) ensured a high poration of AT-PA cells by SLO (see Chapter 4). Table 7. 2 shows the effects of N-SW nuclear extract on the induction of chromosomal aberrations by *Pvu* II (125 units/ml) in AT-PA cells 5 hours after treatment. A reduction in the frequency of *Pvu* II-induced chromosomal aberrations by the nuclear extract was observed in AT-PA cells in two independent experiments when compared to those observed in cells treated with *Pvu* II and BSA (HBSS/BSA) (Table 7. 2). When AT-PA nuclear extract was introduced into AT-PA cells, no such reduction in the yield of *Pvu* II-induced chromosomal aberrations was observed (Table 7. 2). It was noticed that T_4 ligase (50 units/ml), when introduced into AT-PA cells, was associated with an increased number of chromosomal aberrations (Table 7. 2), which is consistent with previously observed results (see Chapter 5).

Table 7. 2. Frequency of chromosome aberrations (CA) per cell induced by *Pvu* II (125 units/ml) following the introduction of nuclear extracts (N-SW or AT-PA cells) into AT-PA cells by SLO (0.3 units/ml) poration.

Treatment	Metaphases scored	% damaged AT-PA cells	Chromosome exchanges	Chromosome deletions	Chromatid exchanges	Chromatid deletions	Chromatid gaps	% heavily damaged cells[a]	CA per cell	Ratio[b]
Exp 1										
HBSS/BSA (-*Pvu* II)[c]	100	26	0	0.05	0	0.11	0.16	0	0.32	-
Buffer C (-*Pvu* II)	100	29	0	0.03	0	0.25	0.12	0	0.40	-
HBSS/BSA (+*Pvu* II)[d]	100	52	0	0	0.01	1.82	0.65	9	2.48	1.00
Buffer C (+*Pvu* II)	100	43	0	0.01	0.01	1.57	0.48	7	2.07	0.81
N-SW extract (+*Pvu* II)	100	22	0	0.03	0	0.52	0.20	2	0.75	0.19

[a]. Chromosome aberrations in heavily damaged cells were not scored and were not included in the number of CA per cell.

[b]. Number of CA per cell (after substraction of background[c]) as a ratio of that observed in RE-treated control[d].

[c]. Background control.

[d]. RE-treated control.

Table 7. 2 (continue). Frequency of chromosome aberrations (CA) per cell induced by *Pvu* II (125 units/ml) following the introduction of nuclear extracts (N-SW or AT-PA cells) into AT-PA cells by SLO (0.3 units/ml) poration.

Treatment	Metaphases scored	% damaged AT-PA cells	Chromosome exchanges	Chromosome deletions	Chromatid exchanges	Chromatid deletions	Chromatid gaps	% heavily damaged cells[a]	CA per cell	Ratio[b]
Exp 2										
HBSS/BSA (-*Pvu* II)[c]	100	34	0	0.02	0	0.06	0.35	0	0.45	-
HBSS/BSA (+*Pvu* II)[d]	150	38	0	0.03	0	0.45	0.51	5	1.02	1.00
N-SW extract (+*Pvu* II)	125	35	0	0.02	0	0.24	0.24	1	0.52	0.12
AT-PA extract (+*Pvu* II)	150	53	0	0.06	0.01	0.31	0.57	1	1.01	0.98
T4 ligase (+*Pvu* II)[e]	100	36	0	0.05	0	0.63	0.47	8	1.20	1.31

[a]. [b]. [c]. [d]. As previous defined.

[e]. T_4 ligase concentration: 50 units/ml.

A comparison of the effects of different treatments on the frequency of chromosomal aberrations induced by *Pvu* II is summarised in Figure 7. 4. The introduction of N-SW nuclear extract resulted in excess of an 80% reduction in chromosomal aberrations in AT cells.

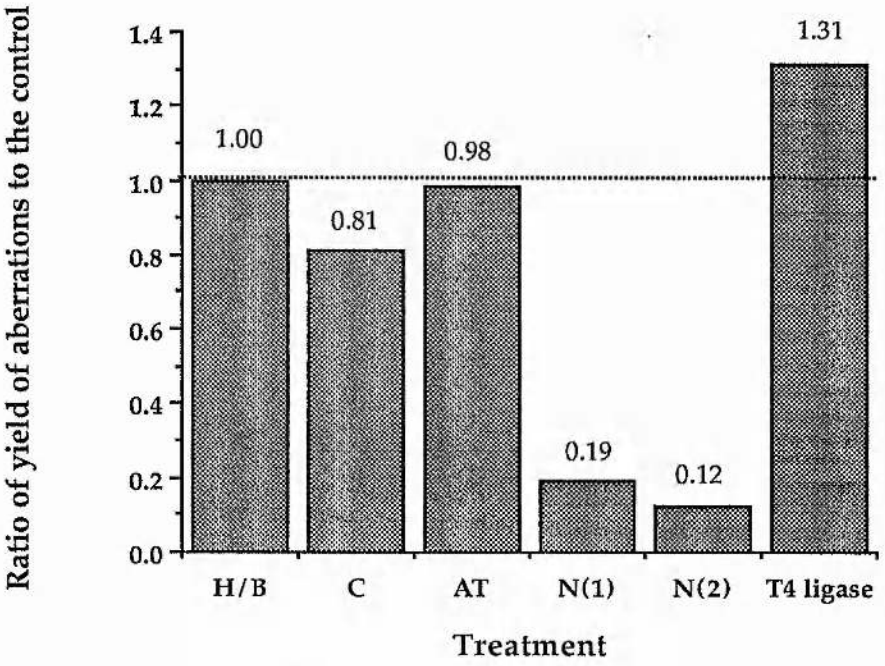

Figure 7. 4. Comparison of the ratios of yield of *Pvu* II-induced chromosomal aberrations to the control (in the presence of HBSS/BSA). H/B: HBSS/BSA; C: buffer C; AT: nuclear extract from AT-PA cells; N: nuclear extract from N-SW cells. The number in the brackets indicates replicate experiments.

7. 3. 3. Assay of *Pvu* II activity following incubation with nuclear extract

The activity of *Pvu* II in the digestion of pBR322 was examined after the incubation of *Pvu* II with nuclear extract. Following purification (see section 2. 2), 5 µl (10 units/µl) of *Pvu* II were mixed with 2 µl of nuclear extract (approximately 2 µg protein) from either AT-PA or N-SW cells, or 2 µl of HBSS/BSA. The mixture was incubated at 37 °C for 4 hours and a titration assay of enzyme activity was carried out as previously described

(section 2. 3). The results are presented in Figure 7. 5. The undigested circular pBR322 appeared at 0.25 units of *Pvu* II under all of the incubation conditions, although the amount of undigested DNA was less in HBSS/BSA at this enzyme concentration. These results indicate that the incubation of *Pvu* II with the extract did not cause a significant reduction in the activity of the enzyme.

7. 3. 4. Effects of normal nuclear extract on the yield of chromosomal aberrations induced by γ-rays

Following the introduction of the normal or AT nuclear extracts into AT-PA cells, the cells were irradiated with 0.3 Gy γ-rays and the chromosomal aberrations analysed 4 hours post irradiation. As shown in Table 7. 3, neither the normal nor AT nuclear extract markedly reduced chromosomal sensitivity of AT-PA cells to γ-rays.

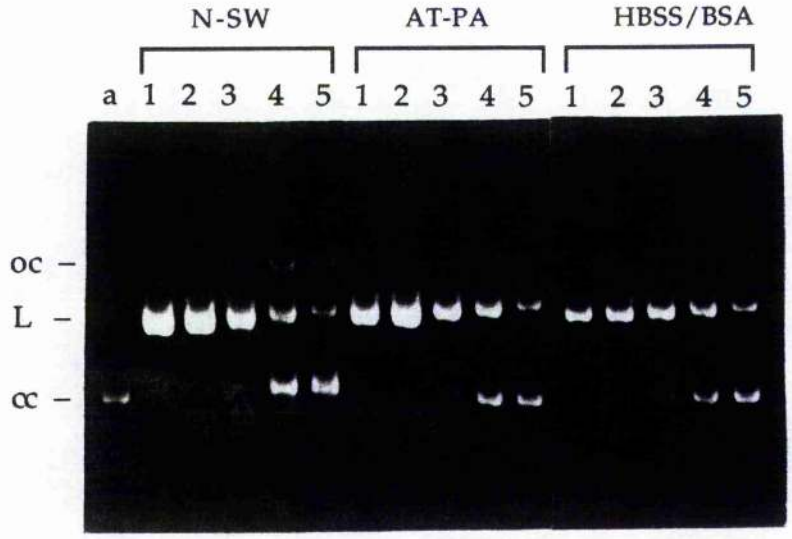

Figure 7. 5. Assay of *Pvu* II activity following incubation with HBSS/BSA or nuclear extracts from AT-PA or N-SW cells as indicated at 37 °C for 4 hours. a: pBR322. Lanes 1 to 5: *Pvu* II 0.5, 0.25, 0.125, 0.06 and 0.03 units, respectively. cc: open circles; L: linear; oc: open circles.

Table 7. 3. Frequency of chromosome aberrations (CA) per cell induced by 0.3 Gy γ-irradiation following the introduction of nuclear extracts (N-SW or AT-PA cells) into AT-PA cells by SLO (0.3 units/ml) poration. 100 metaphases were scored unless otherwise indicated.

Treatment[a]	% damaged AT-PA cells	Chromsome exchanges	Chromosome deletions	Chromatid exchanges	Chromatid deletions	Chromatid gaps	% heavily damaged cells	CA per cell
HBSS/BSA	86	0	0.02	0	1.46	0.66	0	2.14
Buffer C	90	0	0.01	0.02	1.63	0.52	0	2.18
AT-PA extract[b]	84	0	0.01	0	0.68	0.20	0	1.78
N-SW extract	89	0	0.05	0	1.64	0.57	0	2.26

[a]. Background frequency of CA is the same as in Table 7. 1 (continue).

[b]. 50 metaphase were scored.

7. 3. 5. *In vitro* assays of DNases activity in cell extract

The cell extract from N-SW cells was prepared and fractionated by phospho-cellulose chromatography. The extracts were found to have a high DNases activity sufficient to completely digest pBR322 when incubated with the plasmid overnight (data not shown). The extracts were subsequently fractionated in order to remove the nuclease activity. In the first experiment, four fractions of cellular extract were obtained by stepwise elution with 50, 125, 200 and 300 mmol/l KPO_4, respectively (marked as F_{50}, F_{125}, F_{200}, F_{300}, respectively). Approximately 20 -30 ml eluates were obtained with 50 mmol/l KPO_4 elution. Other fractions were approximately 5 -10 ml in volume. After $(NH_4)_2SO_4$ precipitation, dialysis and ultrafiltration, 90 to 200 µl of extract protein were obtained (Table 7. 3). 9 µl of each fraction was incubated with pBR322 0.35 µg at 37 °C for 6 hours. Figure 7. 6 shows that the nuclease activities (apparently both endo- and exonucleases) were mainly found in F_{100}, resulting in a complete digestion of either circular (lane 5) or linear plasmid (lanes 10). Supercoiled (closed circles) plasmid was nicked (resulting in open circles) or linearized (resulting in linear plasmid) by incubation with F_{50} and F_{250} (lanes 4 and 6 in Figure 7. 6), while linear plasmid was not digested (lanes 9 and 11 in Figure 7. 6), indicating an endonuclease activity was present in these fractions. Topoisomerase activity was observed in F_{300} (lane 7 in Figure 7. 6).

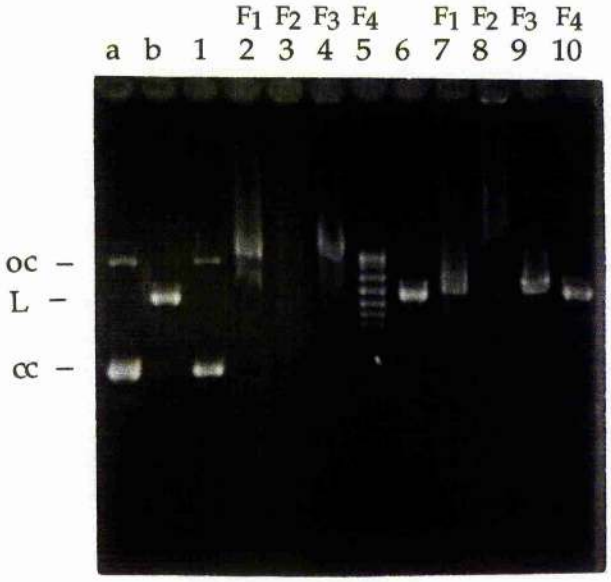

Figure 7. 6. *In vitro* assay of fractions of whole cell extracts. Lane a: pBR322; lane b: linear pBR322. Lanes 1 to 5: circular pBR322 without (lane 1) or with N-SW extract fractions (lanes 2 - 5). Lanes 6 to 10: linear pBR322 without (lane 6) or with N-SW extract fractions (lanes 7 - 10). F_1 to F_4: F_{50}, F_{125}, F_{200} and F_{300}, respectively. cc: open circles; L: linear; oc: open circles. Gel was run in the absence of EB and stained with EB following electrophoresis.

In a second experiment, the extract from N-SW cells was chromatographed and 6 fractions obtained. The topoisomerase activity appeared in F_{200} and F_{300} (lanes 5 and 6 in Figure 7. 7A). In Figure 7. 7A, a DNA nicking activity was observed in F_{50} (lane 4), F_{75} (lane 7) and F_{125} (lane 9), while linear plasmid was not digested by these fractions (lanes 1, 2 and 4 in Figure 7. 7B). It was demonstrated by the denaturation of nicked DNA by formamide at 60 °C that these fractions introduced one or two breaks in the circular plasmid, resulting in an open and linear plasmid respectively (data not shown). High activity of DNases were found in F_{100} and completely digested either circular (lane 8 in Figure 7. 7A) or linear plasmid (lane 3 in Figure 7. 7B) when incubated with the plasmid at 37 °C for 5 hours.

7. 3. 6. Effects of the fractions of whole cell extract from normal cells on the chromosomal sensitivity of AT-PA cells to γ-rays

Fractions of normal extract protein were introduced into either AT-PA or N-SW cells by SLO (0.06 units/ml) poration, followed by 0.3 Gy γ-irradiation. The frequencies of chromosomal aberrations 4 hours post-irradiation are shown in Table 7. 4. As was found with nuclear extract, none of the fractions significantly reduced the frequency of radiation-induced chromosomal aberrations. The poration of normal extract into normal cells had no effect on the induction of chromosomal aberrations by irradiation.

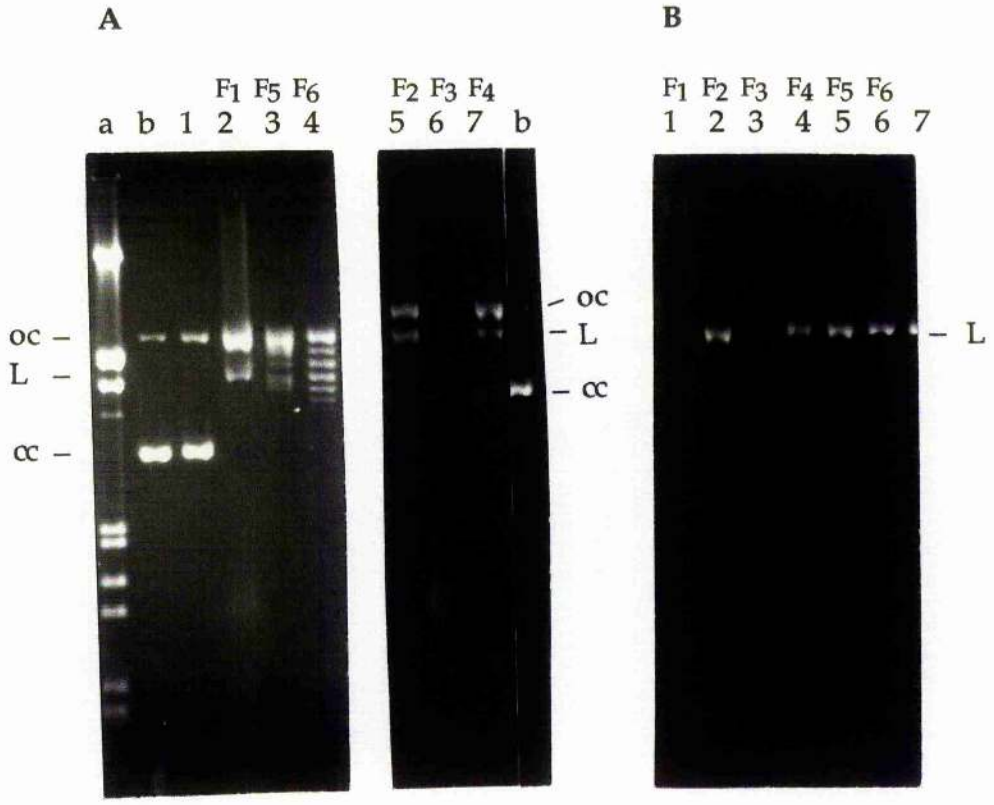

Figure 7. 7. *In vitro* assay of fractions of cell extracts. (A) Incubation of circular pBR322 (0.3 μg) with normal extract. Lane a: lambda DNA digested with *Hind* III and *Eco*R I (marker). Lane b: pBR322. Lanes 1 - 7: pBR322 without (lane 1) or with N-SW extract fractions (lanes 2 -7). (B) Incubation of linear pBR322 without (lane 7) or with N-SW extract fractions (lanes 1 - 6). F_1 to F_6: F_{50}, F_{75}, F_{100}, F_{125}, F_{200} and F_{300}, respectively. cc: open circles; L: linear; oc: open circles. Gels were run in the absence of EB and were stained with EB following electrophoresis.

Table 7. 4. Chromosomal aberrations (CA) per cell induced by γ-irradiation following the introduction of fractions of normal whole cell extract into AT-PA or N-SW cells by poration with SLO (0.06 units/ml). 100 (Exp. 1) or 50 (Exp. 2) metaphases were scored.

Fraction	γ-ray (Gy)	% damaged AT-PA cells	% damaged N-SW cells	CA per AT-PA cell	CA per N-SW cell
Exp. 1					
none	0	19	13	0.24	0.13
none	0.3	82	48	1.87	0.74
F_{50}	0.3	81	36	1.68	0.42
F_{125}	0.3	80	32	1.39	0.43
F_{200}	0.3	80	32	1.82	0.38
F_{300}	0.3	75	41	1.60	0.56
Exp. 2					
none	0	18	12	0.18	0.12
none	0.3	76	38	1.58	0.50
F_{50}	0.3	74	40	1.30	0.50
F_{75}	0.3	90	46	1.74	0.66
F_{100}	0.3	78	-	1.48	-
F_{125}	0.3	82	34	1.38	0.44
F_{200}	0.3	86	52	1.72	0.66
F_{300}	0.3	80	52	1.24	0.74

7. 3. 7. DNA-protein binding in AT-PA and N-SW cell extracts

DNA-protein binding was examined in crude cellular extract from AT-PA or N-SW cells by the method of filter retention as a modification of that described by Mohamed and Lavin (1989). The assay system contained 3 μg of extract protein, 0.2 ng ^{32}P-labelled calf thymus DNA and 0 - 10 μg calf thymus DNA in a total volume of 30 μl. BSA (3μg) was used as a background control, the binding of which was found not to change

significantly with the different amounts of DNA (25 - 200 μg/ml). The background cpm was approximately 10% of the cpm for the samples with extracts. The DNA binding activity of histone proteins (as a pasitive control) at 200 μg/ml of DNA was determined to be approximately 190% and 250% of those for AT-PA and N-SW extracts, respectively.

Figure 7. 8 shows that DNA-protein binding activity is similar between AT and normal extracts at lower concentrations of DNA (below 100 μg/ml), indicating that the initial rate of DNA-protein binding is not significantly different between the two extracts. At higher concentrations of DNA, however, AT extract exhibited higher DNA-protein binding activity than normal extract, implying that a higher amount of DNA-binding proteins may exist in AT extract compared with normal extract (Figure 7. 8).

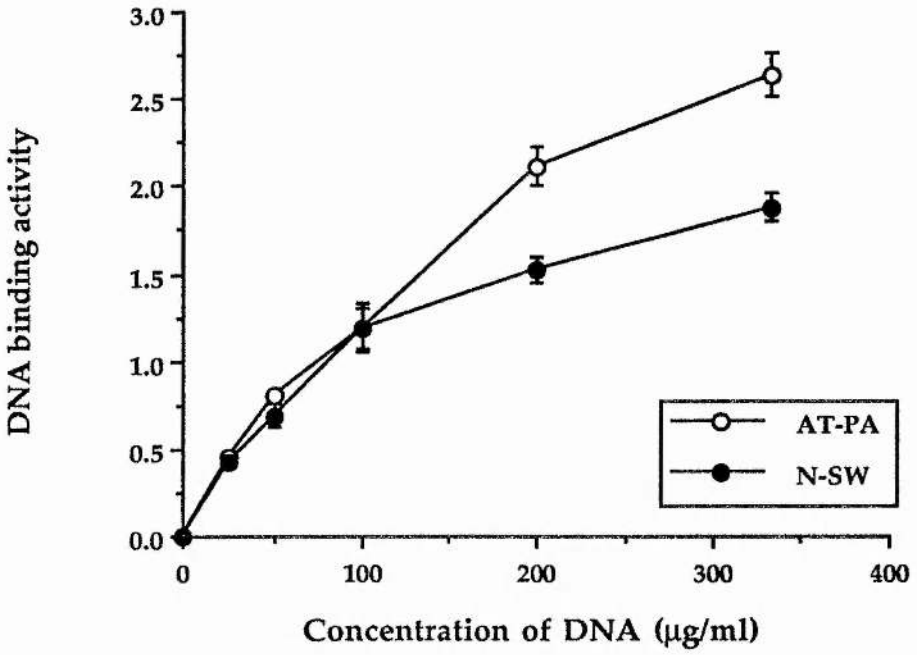

Figure 7. 8. DNA binding activity of cell extract protein as a function of concentration of DNA. Vertical bars represent standard errors of mean values obtained from 3 independent experiments with one preparation of each AT and normal cell extract.

7. 4. Discussion

The data presented here show that nuclear extract derived from normal human lymphoblastoid cells (N-SW), when porated into AT-PA cells, lead to a reduction in the frequency of chromosomal aberrations induced by *Pvu* II in AT cells (Table 7. 2 and Figure 7. 4). Introduction of BSA (in HBSS/BSA) or the nuclear extract from AT-PA cells into AT-PA cells had no such effect (Table 7. 2 and Figure 7. 4). In contrast, neither nuclear extract nor cell extract derived from normal cells influenced the yield of chromosomal aberrations induced by γ-rays in AT-PA cells (Table 7. 3). The results suggest that normal nuclear extract exerts an effect on the processing of dsb generated by RE in AT cells differently from the processing of DNA damage induced by ionizing radiation.

Effects of normal nuclear extract on Pvu II-induced chromosomal aberrations in SLO porated AT cells

Poration of nuclear extract from N-SW cells into AT-PA cells by SLO (0.3 units/ml) reduced the yield of *Pvu* II-induced chromosomal aberrations in AT cells to normal levels (Table 7. 2 and Figure 7. 4) as compared with N-SW cells treated with 0.06 units/ml of SLO and 100 units/ml of *Pvu* II (Table 4. 3 in Chapter 4). Although the comparison was made between different SLO concentrations, it is proposed that the poration efficiency and so the uptake of RE in AT cells at the higher SLO concentration (0.3 units/ml) was comparable to that in N-SW cells porated at the lower SLO concentration (0.06 units/ml), since AT-PA cells were 5 - 6 fold less sensitive to SLO poration than N-SW cells (Chapter 4). In addition, exposure of N-SW cells to 0.3 units/ml of SLO caused severe cell lysis, therefore it was impossible to compare the level of chromosomal aberrations between AT-PA and N-SW cells at the high SLO concentration.

The stability and activity of *Pvu* II during treatment was of special concern. Any reduction in the activity of the enzyme caused by incubation with the extract, presumably as a result of the presence of proteases, could lead to a decreased chromosome breaking effect of RE. *In vitro* assay of the digestion of pBR322 by *Pvu* II showed the enzyme activity was not affected by the incubation of the enzyme with nuclear extract at 37 °C for 4 hours (Figure 7. 3). This result suggests the reduction of RE-induced chromosomal aberrations by normal nuclear extract in AT cells is not caused by a reduced RE activity but is probably associated with the ability of normal nuclear extract to repair the RE-induced dsb more efficiently. However, it is still obscure whether the chromosome cutting ability of *Pvu* II inside cells was influenced by the possible interaction of nuclear extract with chromatin.

The factor(s) in the normal nuclear extract which contribute to the restoration of *Pvu* II-induced chromosomal aberrations in AT cells to a normal level are of interest. Under the present experimental conditions, the break rejoining activity was not detected in the nuclear extract assayed with RE-linearized plasmid, although the extracts were prepared and the rejoining activity assayed under similar conditions to those previously described by North et al (1990) and Fairman et al (1992). These authors reported an efficient rejoining of RE-cut dsb in plasmid by human fibroblast nuclear extract. This unexpected observation implies that the factor(s) which reduced the clastogenetic effects of *Pvu* II in AT cells is unlikely to involve a ligase activity. In fact, T_4 ligase, while showing efficient ligation activity on either blunt- or cohesive-ended dsb in plasmids *in vitro* (Figure 7. 2), did not cause a decrease in the level of chromosomal aberrations when introduced into AT-PA cells, rather it increased the number of aberrations (Table 7. 2), a result previously observed in Chapter 5 (Table 5. 8).

The activity of ATP-independent topoisomerase (Topo I) that causes a relaxation of supercoiled plasmid was observed in nuclear extract (Figure 7.

2). Topo II activity, which would convert supercoiled circular pBR322 to contenanes (join by strand passing) in the presence of ATP was not measured under the present assay conditions. No obvious nuclease activity was determined *in vitro* in the nuclear extract using plasmid (Figure 7. 3). In the absence of SDS, a DNA-binding activity of proteins in the nuclear extract was observed *in vitro*, leading to a lagging migration of plasmid DNA during electrophoresis (data are not shown).

A comparison of the DNA-protein binding capacity in cell extract from N-SW and AT-PA cells revealed a difference in DNA-protein binding between the two extracts; AT extract showed higher DNA-protein binding activity than normal extract (Figure 7. 8). This result is inconsistent with the data reported by Mohamed and Lavin (1989) who showed that the extent of binding with normal cell extract was approximately 2-fold higher than that for cell extract from AT lymphoblastoid cells (AT2ABR). In the experiment of Mohamed and Lavin (1989), a constant amount of ^{32}P-labelled DNA (10 μg plasmid DNA inserted sequence of histone genes) was incubated with varying amount of cell extract, while in this experiment DNA-protein binding activity was determined as a function of concentration of DNA. Binding of protein to the trace amount of ^{32}P-labelled DNA was inversely related to the amount of cold DNA added. On the other hand, calf thymus DNA was used in the present study so that the capacity of protein in binding whole sequence mammalian DNA, rather than histone genes alone was investigated. The significance of the higher activity of DNA-protein binding observed in AT extract compared with normal extract is not clear. Nevertheless, the result indicates a difference in DNA-protein binding activity between AT and normal extracts, although the experiment needs to be repeated again with different preparations of extract.

Chromosomal instability of AT cells to DNA damage was suggested to be due to a defect in processing DNA damage in AT cells. The only direct

evidence of such a defect existing in AT cells was derived from a set of studies using RE-broken vector DNA to model dsb-rejoining in transformed AT and normal cells (reviewed by Thacker 1989). A lower fidelity of rejoining of RE-cut dsb in a selectable gene in vector DNA was consistently observed in AT cells when compared with normal cells (Cox 1984, Cox et al 1986, Debenham et al 1988, Green and Lowe 1987, Thacker 1989, Powell et al 1992, Rünger et al 1992). Furthermore, a higher than normal frequency of misrejoining of enzyme-restricted plasmid has also been demonstrated in AT nuclear extract (North et al 1990, Ganesh et al 1993). The elevated frequency of mis-repair of dsb of plasmid in AT cells was thought to be due to either an altered exonuclease activity in AT cells or a lack of protection in the broken ends of DNA strand (Cox et al 1986, North et al 1990, Thacker et al 1993).

From the findings that RE-induced chromosomal aberrations in AT and normal cells were potentiated by ara A (Chapter 5), it is plausible to postulate that strand exposure is a crucial step in the repair of RE-induced dsb. An inefficient end protection may lead to an extensive degradation of DNA strands by exonuclease. The end protection seems to be important for the initial step of repair of RE-induced dsb. North et al (1990) has proposed that the nuclear extract from normal cells protect the strand exposure to exonuclease more efficiently than AT nuclear extract. It may be assumed that such proteins which exert DNA strand end protection in normal human extract, once introduced into AT cells, would assist the alignment of broken strands and prevent otherwise deleterious strand exposure in AT cells. Therefore, the functional factors in normal extract which resulted in a reduced chromosomal sensitivity of AT cells to RE may involve proteins that affect the stability or organisation of chromatin structure in AT cells.

Effects of nuclear and cell extracts on chromosomal aberrations induced by γ-rays in porated AT-PA cells

The results showed an inability of normal nuclear or cellular extract to restore γ-irradiation-induced chromosomal aberrations in AT cells (Table 7. 3 and 7. 4). Both the proportion of AT-PA cells containing chromosomal damage and the yield of chromosomal aberrations induced by irradiation were not markedly changed after poration of normal extract. Poration of cells by SLO (0.06 units/ml) did not significantly affect the induction of chromosomal aberrations by irradiation, as observed in irradiated N-SW cells with or without treatment with normal extract (Table 7. 4).

Although several enzyme activities, e.g., nuclease (exo- or endonuclease) and topoisomerase were detected in the nuclear extract or the fractions of cell extract derived from N-SW cells, neither nuclear extract nor fractions of cellular extract influenced the yield of chromosomal aberrations in irradiated AT cells. These data indicate that the presence of nuclease in the extract had only minor effects on chromosomal aberrations. The activity of topoisomerase appeared to have no effect on the repair of chromosomal damage by γ-rays.

Two possible explanations for these results for γ-irradiated cells may be made. It was noticed that with 0.3 units/ml SLO poration, in most cases less than 40% of cells were found to contain aberrations induced by *Pvu* II (Table 7. 2). This might point to an approximate porated cell population. The cutting of chromosomal DNA occurs only in those porated cells following entry of enzyme into cells. In contrast, in the case of irradiation, chromosomal damage was found in 70 - 90 % of cells. Nearly half of this cell population were presumably not porated and therefore probably no extract could have entered these cells. Therefore, any change in the frequency of aberrations following radiation might not be significant because of the large population of damaged cells which are not porated.

The second explanation may be that normal extract is not able to confer a normal chromosomal sensitivity to irradiation in AT cells. It is known that radiation-induced DNA breaks are more complicated than RE-induced damage with respect to end-structure, thereby more complicated repair mechanisms for radiation-induced DNA damage than for RE-induced dsb may be required. The normal extract obtained may not include all of these functions thus could not complement the defective repair mechanism in AT cells.

Conclusions

AT lymphoblastoid cell lines, AT-PA and AT-KM, which exhibited a 3-fold hypersensitivity to ionizing radiation, also showed a 2 - 4 fold enhanced chromosomal sensitivity to dsb caused by RE when compared with a normal lymphoblastoid cell line (N-SW). The production of chromosomal aberrations by RE was found to mimic the cytogenetic effects of radiation in that chromatid aberrations are induced in G_2 cells (harvested 5 h after RE treatment) and both chromosome- and chromatid-type aberrations in G_1 cells (harvested 24 h after RE treatment). In AT cells, the increased chromosomal sensitivity to RE of G_1 cells (harvested 24 hours after RE treatment) was predominantly in the form of chromatid aberrations and this is in agreement with the observations of AT cells following G_1 irradiation. Elevated yields of chromosomal aberrations induced by *Pvu* II occurred in AT-PA cells in spite of reduced induction of dsb by *Pvu* II found in the AT cells compared to N-SW cells, a result which corresponds with the lower poration efficiency by SLO observed in AT-PA than N-SW cells. The data suggest a dsb processing defect in AT cells which converts a higher number dsb into chromosomal aberrations than is the case for the normal N-SW cell line.

RE which generate cohesive-ended dsb (*Bam*H I and *Pst* I) were found to be less clastogenic than RE which generate blunt-ended dsb (*Pvu* II and *Eco*R V) in both AT and normal cell lines. These results are consistent with previous observations that cohesive-ended dsb induced by RE are less effective than blunt-ended dsb for a number of biological end-points. *In vitro* assay of RE activity with plasmid DNA showed that RE activities in simulated cellular conditions were quite different which may partly, but not absolutely, account for the different clastogenetic effects of RE. The different efficiencies of induction of chromosomal aberrations by RE may also reflect

a difference between the processing of dsb with cohesive ends and those with blunt-ends.

Both AT cell lines (AT-PA and AT-KM) exhibited a higher frequency of aberrations arising from cohesive-ended dsb induced by either *Bam*H I or *Pst* I when compared with normal cells. This result indicates that AT cells are also defective in processing dsb with cohesive termini.

The induction of G_2 chromatid aberrations induced by either γ-irradiation or by *Pvu* II was potentiated by ara A in AT as well as in normal cells. The result indicates that RE-induced dsb are probably subject to end degradation to certain degree, and that the repair of RE-induced dsb may not only involve some direct ligation but also mechanisms requiring DNA repair synthesis. Although the extent of potentiation of aberration frequencies by ara A in AT-KM and AT-PA cell lines was not significantly different from that in the normal N-SW cell line following irradiation at G_2 phase, AT cells were shown to be influenced by ara A to a lesser extent than normal cells after treatment with *Pvu* II and *Pst* I. The different response to ara A of AT and normal cells is unlikely to be explained by a reduced level of degradation at the ends of dsb in AT cells, but may indicate an already extensive end degradation in AT cells thereby making AT cells insensitive to ara A. In view of the fact that ara A had no effect on the frequency of chromosomal aberrations induced by *Bam*H I, the putative degradation at the broken dsb ends was postulated to depend somewhat on the end-structure of the lesion; i.e., blunt- and 3'-overhang cohesive-ended dsb are more likely to be degraded than 5'-overhang cohesive dsb.

RE-induced blunt-ended dsb induced an inhibition of DNA synthesis in normal lymphoblastoid cells, indicating that this type of dsb may be one of the main lesions responsible for the reduction of the rate of DNA synthesis observed after ionizing radiation. In contrast, AT-PA cells were

shown to be resistant to RE with regard to DNA synthesis, a response which is similar to that observed following ionizing radiation.

Pvu II-induced chromosomal aberrations were restored to a normal level in AT-PA cells by poration of nuclear extracts derived from normal (N-SW) cells. This effect appeared not to be a result of action of the ligase. Moreover, it was also shown that T_4 ligase did not reduce the frequency of chromosomal aberrations induced by *Pvu* II after being introduced into AT-PA cells or N-SW cells. However, neither nuclear nor whole extracts of normal cells could confer a normal chromosomal sensitivity on AT-PA cells exposed to ionizing radiation. The findings suggest that the factor(s) in normal nuclear extracts which are able to complement the defect in AT cells when treated with RE may be involved in preventing the deterioration of strand ends by exposure to exonuclease in AT cells.

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APPENDIX

Published articles

1. **Liu, N. and Bryant, P.E. (1993)** Response of ataxia-telangiectasia cells to restriction endonuclease induced DNA double strand breaks: I. Cytogenetic characterization, *Mutagenesis*, **8**, 503-510.

2. **Liu, N. and Bryant, P.E. (1993)** Hyper-sensitivity of ataxia telangiectasia cells to DNA double strand breaks induced by restriction endonuclease, *Abstracts of 25th Annual Meeting of the European Society for Radiation Biology*, June 10-14, 1993, Sweden, pp L07:09.

3. **Liu, N. and Bryant, P.E. (1994)** Enhancement of restriction endonuclease caused chromosomal aberrations by 9-β-arabinofuranosyladenine in normal human and ataxia telangiectasia lymphoblastoid cells, *International Journal of Radiation Biology*, **65**, 138-139.

4. **Bryant, P.E., Jones, N.J. and Liu, N. (1993)** Radiosensitive Chinese hamster *irs* 2 cells show enhanced chromosomal sensitivity to ionising radiation and restriction endonuclease induced blunt-ended double strand breaks, *Mutagenesis*, **8**, 141-147.

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Response of ataxia telangiectasia cells to restriction endonuclease induced DNA double-strand breaks: I. Cytogenetic characterization

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Ataxia telangiectasia (AT) and normal human lymphoblastoid cell lines have been treated with either X-rays or the restriction endonucleases *Pvu*II and *Bam*HI using streptolysin-O poration, and the frequencies of micronuclei or chromosomal aberrations measured. We report that AT cells (AT-PA) are hypersensitive to the restriction endonucleases *Pvu*II and *Bam*HI, inducing DNA double-strand breaks (dsb) with either blunt or cohesive termini, respectively. Our data indicates that AT-PA cells have a dsb processing defect that leads to a higher rate of conversion of dsb into chromosomal aberrations than in normal cells. AT-PA cells showed up to a 5-fold enhanced sensitivity to *Pvu*II over the normal (N-SW) line, a result of an increase in frequencies of chromatid aberrations. Chromosome-type aberrations appeared not to be increased in AT-PA cells over those induced in the normal N-SW line. Particularly striking was the appearance in AT-PA of high frequencies of chromatid aberrations at the 24 h sampling time. *Bam*HI also caused enhanced aberration frequencies in AT-PA cells although the cohesive-ended dsb caused by *Bam*HI still appeared to be *less* effective in causing chromosomal aberrations than the blunt-ended dsb caused by *Pvu*II in both AT-PA and N-SW, as we have previously reported for Chinese hamster cells. The enhanced effectiveness of cohesive-ended dsb in AT-PA cells over normal cells may be a result of altered processing of dsb by AT-PA cells or may be caused by conversion of some cohesive-ended dsb into blunt-ended dsb by exonuclease digestion before ligation can take place.

Introduction

Ataxia telangiectasia (AT) is an human autosomal recessive genetic disease characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, immunodeficiency and predisposition to malignancy (Boder and Sedgwick, 1958; Boder, 1985). Genetic linkage analysis has indicated the location of the AT gene on chromosome 11q22–23 (Gatti *et al.*, 1988; Ejima *et al.*, 1990). Cells of AT individuals show diminished inhibition of the initiation of DNA synthesis following X-irradiation (Houldsworth and Lavin, 1980; Painter and Young, 1980), altered cell cycle response to irradiation (Zampetti-Bosseler and Scott, 1981, 1985), and hypersensitivity to the clastogenic effects of ionizing radiation and other genotoxic agents, e.g. bleomycin (Rary *et al.*, 1974; Taylor *et al.*, 1975; Lehmann and Stevens, 1977; Taylor, 1978; Natarajan and Mayers, 1979; Zampetti-Bosseler and Scott, 1985). AT cells have been shown to exhibit a difference in the response of chromatin supercoiling to radiation (Taylor *et al.*, 1991). Altered DNA binding properties of proteins in cellular extracts of AT cells have been reported, also indicating possible chromatin differences in AT (Mohamed and Lavin, 1989).

AT cells exhibit an enhanced frequency of radiation induced chromosomal aberrations in both G_0 and G_2 phases of the cell cycle (Taylor, 1978, 1982; Bender *et al.*, 1985; Parshad *et al.*, 1985; Mozdarani and Bryant, 1989) and an unusual occurrence of chromatid deletions in cells exposed to ionizing radiation in the G_0 phase attributed to a defect in DNA repair (Taylor *et al.*, 1975; Taylor, 1978). Although the mechanism of chromosomal aberration formation is not fully understood, it was postulated that they arise from dsb (Bender *et al.*, 1974). Using restriction endonucleases to induce dsb in DNA of cells it was demonstrated that the dsb is a DNA lesion which can give rise to chromosomal aberrations (Bryant, 1984; Natarajan and Obe, 1984). However, a number of studies have shown that both induction by radiation and rejoining of bulk dsb in AT cells is normal (Lehmann and Stevens, 1977; Fornace and Little, 1980; Thierry *et al.*, 1985), although one report suggests that the kinetics of repair may be different in AT from that in normal cells (Coquerelle and Weibezahn, 1981).

We have previously reported, using the G_2 assay, that in AT cells an enhanced conversion of radiation induced dsb into chromatid breaks occurs even at very short times after irradiation (Mozdarani and Bryant, 1989). These findings have recently been corroborated (Pandita and Hittleman, 1992) in studies using premature chromosome condensation (PCC), where in contrast to earlier reports (Cornforth and Bedford, 1983, 1985) where equal numbers of initial PCC fragments were induced in AT and normal fibroblasts, a 2-fold higher initial level of chromosome breaks was observed in AT than normal G_1 and G_2 cells.

Using Chinese hamster V79 and Chinese hamster ovary (CHO) cells we showed previously that treatment of permeabilized or porated cells with restriction endonucleases leads to the induction of chromosomal aberrations (Bryant, 1984; Bryant and Christie, 1989), cell killing (Bryant, 1985), cell mutation (Singh and Bryant, 1991), and oncogenic transformation (Bryant and Riches, 1989). Enzymes such as *Pvu*II (inducing blunt-ended dsb) were found to be much more effective than those inducing dsb with cohesive termini and 4 base overlap (such as *Bam*HI) in producing chromosomal aberrations (Bryant, 1984; Bryant and Christie, 1989; Moses *et al.*, 1990). This effect seems to correlate with a lack of accumulation in cellular DNA of dsb caused by *Bam*HI (Costa and Bryant, 1990) and possibly results from a more rapid repair of cohesive- than blunt-ended dsb due to the base overlap possibly facilitating repair of the two breaks as single-strand lesions.

We have shown that radiosensitive dsb repair deficient xrs5 cells (Jeggo and Kemp, 1983) exhibit elevated expression of chromosomal damage (Bryant *et al.*, 1987) and increased accumulation of dsb following treatment with *Pvu*II (Costa and Bryant, 1991). Also, in a recent study we have shown that the V79 mutant irs2 (Jones *et al.*, 1987, 1990; Thacker and Ganesh, 1990), a line showing many similarities of response to those of AT cells, exhibits chromosomal hypersensitivity to *Pvu*II-induced dsb, manifest as a 2- to 4-fold increased yield of both deletion and exchange-type chromosomal aberrations (Bryant *et al.*, 1993).

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Here we report on experiments in which the chromosomal response of AT and normal lymphoblastoid cells was measured following exposure to the restriction endonucleases *Pvu*II and *Bam*HI during poration with the bacterial cytotoxin streptolysin-O (SLO). Cell poration with SLO was shown to be preferable to electroporation since it resulted in a higher viability of Chinese hamster cells than after electroporation (Bryant, 1992). As we show here, the SLO technique was found to be effective in porating human lymphoblastoid cells.

Materials and methods

Cell lines and culture

Lymphoblastoid cell lines: ataxia telangiectasia (AT-PA) and normal (N-SW) were kindly supplied by Dr A.M.R.Taylor (Department of Cancer Studies, University of Birmingham, UK). These were grown as suspension cultures in RPMI medium with the addition of 10% fetal calf serum, antibiotics and 10% tryptose phosphate broth, in an atmosphere of 5% CO_2. Cells were maintained in exponential growth by routine passage every 3 days between 3×10^5 and 1×10^6 cells/ml. For experiments, cells were passaged 1–2 days previously at 3×10^5 cells/ml.

Irradiation

AT cells were characterized by their cytogenetic response to 250 kV X-rays. Cells were exposed to X-rays (Marconi Therapy set; 0.5 mm Cu filter) in aerated medium at ambient temperature (~20°C) in plastic TC flasks at a dose-rate of about 1 Gy/min. The γ-ray irradiation for dsb measurement was carried out in a ^{137}Cs IBL 437 C γ-irradiator at a dose rate of 4.6 Gy/min on ice. Dosimetry was checked by a ferrous sulphate method (Frankenberg, 1969).

Restriction endonucleases

Restriction endonucleases (*Pvu*II and *Bam*HI; BRL-Life Technologies, Paisley, UK) were purified using Amicon Ultrafiltration (Bryant and Christie, 1989) and diluted to 10 units/μl in Hank's balanced salts solution (HBSS).

Cell poration with SLO

Cell poration was carried out essentially as previously described for Chinese hamster cells (Bryant, 1992). Cells grown in suspension were centrifuged and cell pellets resuspended in HBSS at 1×10^6/ml. SLO (Wellcome Diagnostics, Dartford, UK) was dissolved as per the manufacturers' recommendations, usually giving a stock solution of 1.9 units/ml, which was stored in aliquots (0.5 ml) at −20°C. SLO was added to cells (usually 0.5 or 1 ml) in HBSS at ambient temperature (~20°C) to a final concentration of 0.06 units/ml in the presence of restriction endonucleases. Cells were thus held for 5 min and then 5 ml (5-fold volume) of RPMI medium added and centrifuged. The resultant cell pellet was resuspended gently in fresh RPMI and incubated at 37°C for various times.

Determination of double strand breaks

Cells (5×10^5 cells/ml) were labelled with [^{3}H]thymidine at 3.7 KBq/ml for 16 h. For γ-irradiation, cells were cooled down on ice for 30 min before irradiation. Dsbs were determined immediately. For restriction endonuclease treatment, cells were centrifuged and resuspended in HBSS at 1×10^6 cells/ml. Then, 5×10^5 cells were treated with 0.06 units/ml of SLO and 500 units/ml of *Pvu*II or *Bam*HI in a total volume of 0.5 ml as described above. After restriction endonuclease treatment cells were resuspended in RPMI medium and incubated at 37°C for 4.5 h. Dsbs were measured by neutral filter elution at pH 9.6 (Bradley and Kohn, 1979) as modified by Okayasu and Iliakis (1989).

Cell poration assay

Cells were seeded at 5×10^5 cells/ml in RPMI and incubated with [^{3}H]methionine (Amersham International, Amersham, UK) at 1 μCi/ml (3.7×10^4 Bq/ml) for 24 h. The labelled cells were centrifuged, washed once with RPMI medium and chased in non-radioactive RPMI medium at 37°C for 24 h. The cells were then harvested in HBSS and treated with SLO at various concentrations for 5 min or at 0.06 units/ml of SLO for various times. Cells were pelleted and whole supernatants (0.5 ml each) were loaded on Whatman no. 3 filter discs, dried at 60°C and radioactivity counted by liquid scintillation. In some experiments the supernatants were ultrafiltered with Amicon 10 filters (which trap molecules with mol. wt >10 kDa) and ^{3}H activities in filtrate and on the filter counted.

Cell harvest and cytogenetic preparation

After various incubation times colcemid was added at 0.04 μg/ml for 4 h. Cells were harvested by centrifugation, resuspended in 0.075 M KCl at room temperature for 3 min, recentrifuged and fixed in methanol:acetic acid (3:1). Cells were centrifuged and similarly fixed three more times after overnight storage at 4°C. Cells were dropped on to cooled slides (−20°C) and air dried. Chromosomes were stained in 3% Giemsa in tap water.

Micronucleus assay

Micronuclei were assayed by the cytokinesis block technique (Fenech and Morley, 1985). Cytochalasin B was added at 3 μg/ml to cultures following X-irradiation. Cells were incubated for various times and samples cytospun (Shandon Cytospin 2), air dried, fixed in methanol for 10 min, dried again and stained in filtered 10% Giemsa in tap water. Micronuclei were scored in binucleated cells.

Results

Chromosomal sensitivity to X-rays

Figure 1 shows results of micronucleus induction by 2 Gy of X-rays in AT-PA and N-SW cells sampled at various incubation times after irradiation. AT cells showed a 2.5- and 3.8-fold enhanced yield of micronuclei at 48 h and 72 h sampling times, respectively, over the level in normal cells (Figure 2), indicating the characteristic chromosomal hypersensitivity of AT to ionizing radiation.

Poration assay

To examine the effects of SLO on cell membrane poration, we determined the leakage of [^{3}H]methionine labelled cellular proteins. After poration supernatants were collected and ultrafiltered using Amicon 10 filters. The ^{3}H activities in the filtrate and on the filter were then counted. The results showed that for both AT-PA and N-SW cell lines >80% of radioactivity was retained by the filter, indicating that the majority of labelled molecules were proteins of mol. wt >10 kDa. Figure 3 shows data for leakage of ^{3}H-labelled proteins from AT and N-SW cells following SLO poration. AT-PA cells showed 4- to 5-fold lower leakage of proteins than normal cells in the SLO dose–effect assay (Figure 3a), indicating reduced poration by SLO in this cell line. Experiments using the Trypan blue assay with a graded series of SLO concentrations (data not shown) also indicated a more effective poration of normal than AT cells.

Dsb induced by restriction endonucleases in porated cells

Dsb induced by *Pvu*II and *Bam*HI in porated AT-PA and N-SW cells were quantified using neutral filter elution. Results are shown in Figure 4. *Pvu*II caused a level of DNA elution corresponding to about a 6-fold higher frequency of dsb in N-SW than in AT-PA cells, which was probably a result of more effective poration of N-SW than AT-PA cells (Figure 3). *Bam*HI showed less effectiveness in inducing dsb than *Pvu*II in N-SW

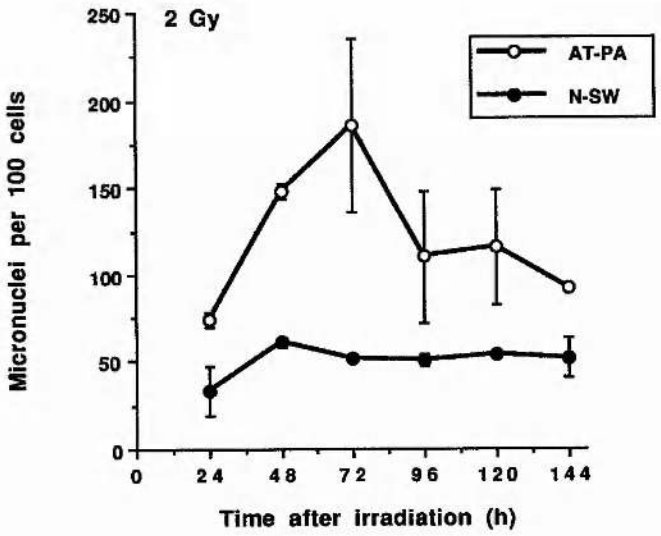

Fig. 1. Frequency of micronuclei in AT-PA (○) and N-SW (●) cell lines irradiated with 2 Gy of X-rays and post-incubated for 24–144 h. Error bars represent standard error of mean values from three independent experiments.

cells, but appeared to be as effective as *Pvu*II in AT-PA cells. The frequency of dsb in *Pvu*II (500 units/ml) treated AT-PA and N-SW cells can be estimated from a γ-ray dose–effect curve (Figure 5) to be equivalent to the dsb induced by ~2 and 10 Gy γ-rays, i.e. ~80 and 400 dsb/cell, respectively (Blöcher, 1982).

Chromosomal response of cells to restriction endonucleases

Figure 6 and Table I show the chromosomal response of AT-PA and N-SW cells to *Pvu*II at 5 and 24 h sampling times. AT-PA cells exhibit up to a 5-fold enhanced sensitivity to *Pvu*II for both sampling times (Figure 6). The percentage of cells of both lines containing aberrations increased proportionally with the concentration of *Pvu*II; the frequency of damaged cells was higher in AT-PA than N-SW cells (Figure 7).

Table I shows that at 24 h more chromatid than chromosome aberrations were observed in AT-PA than in N-SW cells. Increased numbers of chromatid deletions and gaps were observed in both AT-PA and N-SW cells treated with *Pvu*II (Table I). This was more pronounced at 5 than 24 h. AT-PA (but not N-SW) cells showed remarkably high frequencies of chromatid exchanges at both 5 and 24 h. In N-SW cells chromatid exchanges only occurred at higher concentrations of *Pvu*II and at the 5 h fixation time.

Figure 8 and Table II show data for AT and N-SW cells treated with *Bam*HI. This enzyme was found to induce aberrations in AT-PA cells (albeit at a 5-fold lower frequency at 5 h and ~2-fold lower frequency at 24 h) while normal cells showed no significant increase over background levels at either 5 or 24 h. Some increase in frequency of chromatid gaps was observed in AT cells treated with *Bam*HI although not as pronounced as after *Pvu*II.

Discussion

We have shown that restriction endonuclease treatment of porated human lymphoblastoid cells mimics the clastogenic effect of radiation in causing both break and exchange type chromosomal aberrations. Our results show that *Pvu*II induces a higher

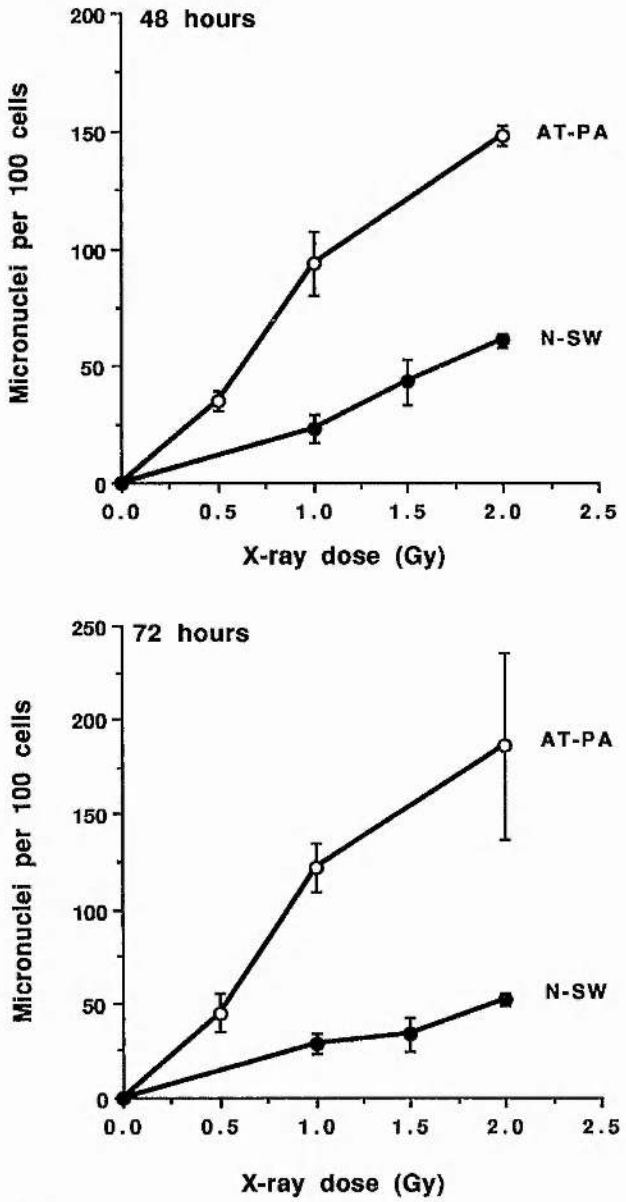

Fig. 2. Dose–effect relationships for micronucleus yields of X-irradiated AT-PA (○) and N-SW (●) cells post-incubated for (a) 48 h and (b) 72 h. Error bars represent standard error of mean values from three independent experiments.

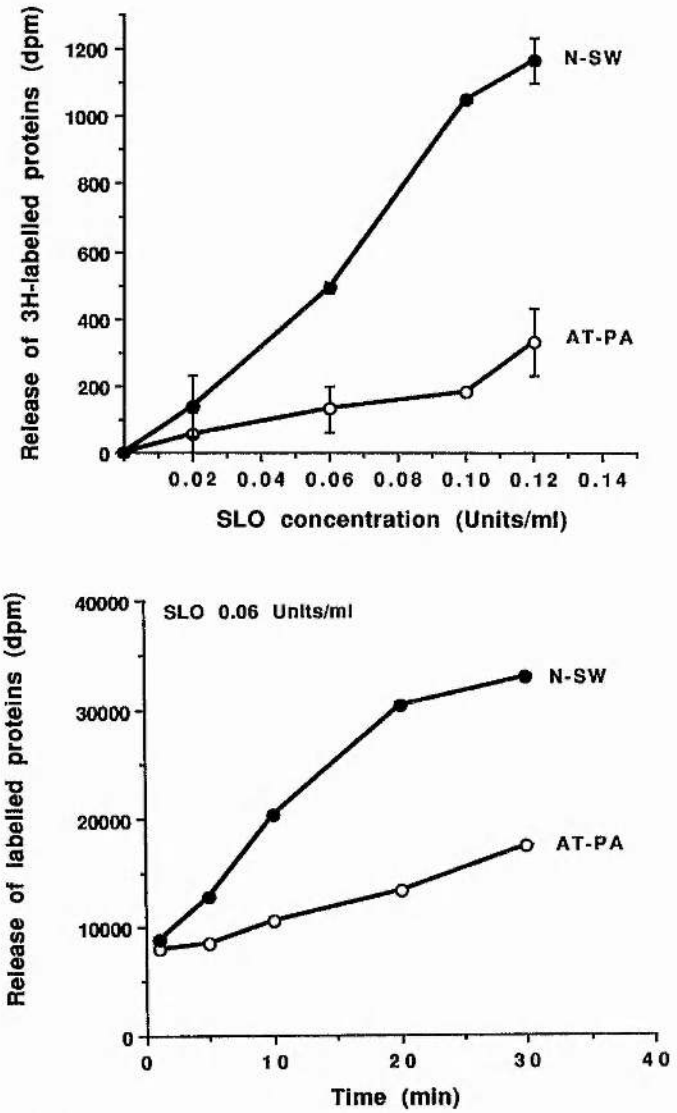

Fig. 3. Leakage of [^{3}H]methionine labelled cellular proteins from SLO porated AT-PA (○) and N-SW (●) cells. (a) Cells exposed to 0–0.12 units/ml SLO for 5 min. (b) Cells exposed to 0.06 units/ml SLO for 1–30 min. Error bars represent standard error of mean values from two or three independent experiments.

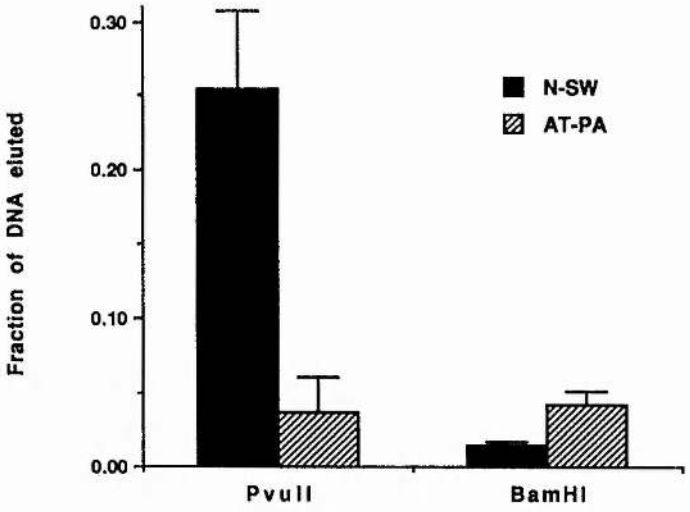

Fig. 4. Dsb induced by *Pvu*II and *Bam*HI (both at 500 units/ml) in SLO (0.06 units/ml) porated AT-PA and N-SW cells after 4.5 h post-treatment incubation. Data are pooled from four (*Pvu*II) and two (*Bam*HI) independent experiments. Vertical bars represent standard errors of mean values.

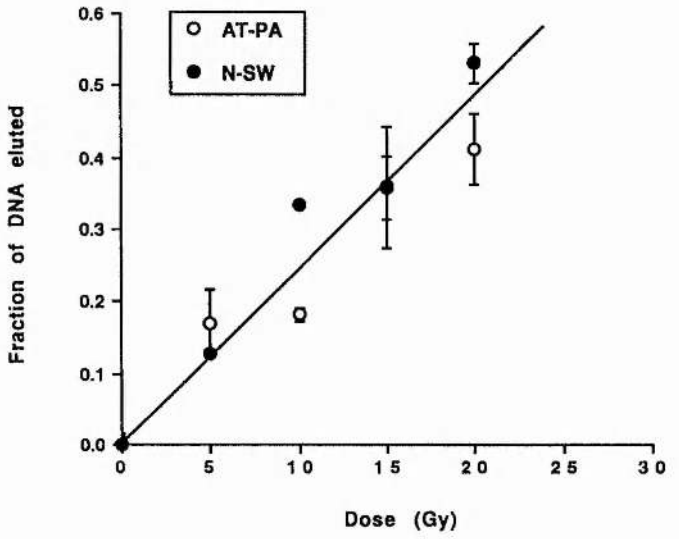

Fig. 5. Induction of dsb as a function of γ-ray dose in AT-PA (○) and N-SW cells (●) immediately after irradiation. Means values and standard errors are from two independent experiments.

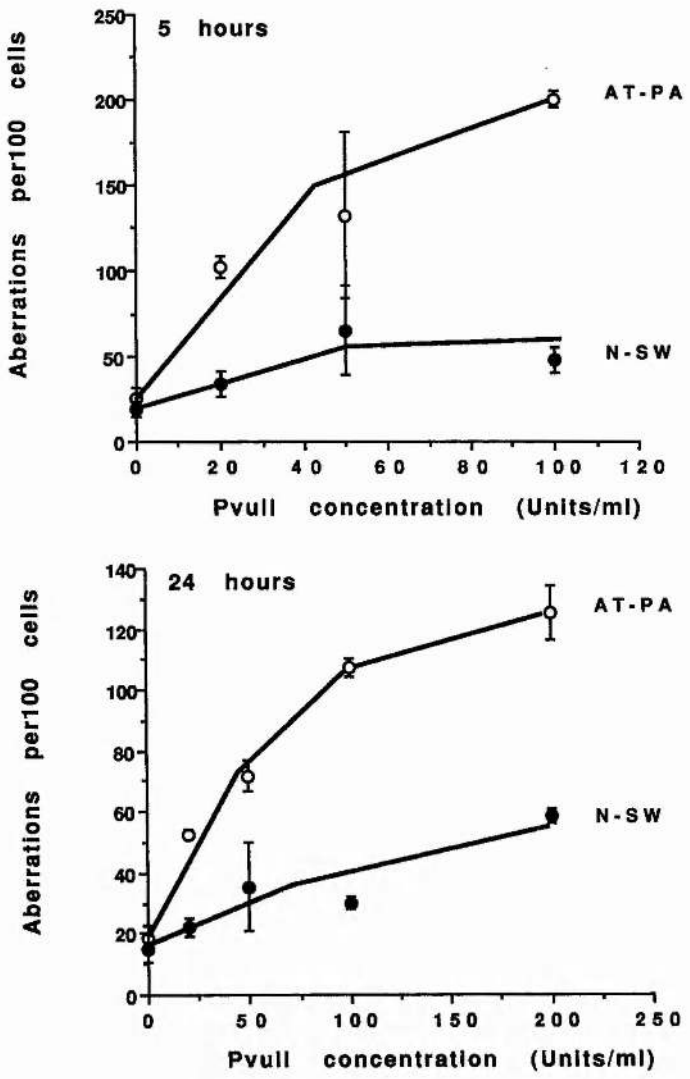

Fig. 6. Frequency of chromosomal aberrations (including gaps) in AT-PA (○) and N-SW (●) cells treated with *Pvu*II for (a) 5 h and (b) 24 h. Error bars represent standard error of mean values (see Table I).

frequency of aberrations in AT than normal cells (Figure 6), although more dsb were induced in N-SW than in AT-PA cells under the poration condition used, indicating that the AT cell line has a defect in the processing of blunt-ended dsb that results in an increased conversion of dsb into chromosomal aberrations. We also show that *Bam*HI, which causes cohesive-ended dsb, induces a higher frequency of aberrations in AT cells (Figure 8).

We have confirmed the intrinsic hypersensitivity of AT-PA cells to X-rays and found they are chromosomally approximately three times more sensitive to X-rays than normal (N-SW) cells (Figure 2), as found for other AT cell lines exposed to ionizing radiation (Taylor, 1978).

Our results from protein leakage experiments indicate that AT-PA cells are approximately five times less efficiently porated by SLO than the N-SW line. The frequency of dsb induced by *Pvu*II under the porating conditions used in our clastogenic analyses (0.06 units/ml of SLO) were found to be ~6-fold lower in AT-PA than in N-SW cells, reflecting the less efficient poration measured by protein leakage. We thus suggest that the lower production of dsb in AT-PA cells is a result of less efficient poration by SLO. This is supported by the fact that in the AT line, the frequency of dsb can be increased by increasing SLO concentration (data are not shown). However, we were unable to increase the SLO concentration in AT cells in our cytogenetic experiments since the mitotic index of cells treated at concentrations in excess of 0.06 units/ml fell to an unacceptable level. These observations introduce some uncertainty into our conclusions, but suggest that the frequency of aberrations for a given concentration of restriction endonuclease in AT-PA might have been even higher than in N-SW had the poration been exactly equivalent in the two cell lines.

At 5 h after *Pvu*II treatment (Figure 6a) only chromatid-type aberrations (breaks, gaps and exchanges) were found in AT-PA and N-SW lines, and the total number of aberrations was higher than that at the 24 h sampling time (Figure 6b). A striking result was that at the 24 h sampling time AT-PA cells showed high levels of chromatid aberrations compared with N-SW, whereas the frequencies of chromosome aberrations at 24 h were not different in the two lines (Table I).

Table I. Metaphase aberrations per 100 AT-PA or N-SW cells treated with *Pvu*II

Cell line	Units/ml	Fixation time (h)	No. of cells analysed[a]	Percent of damaged cells	Chromosome exchanges	Chromosome deletions	Chromatid exchanges	Chromatid deletions	Chromatid gaps	Total aberrations[b]	SEM
AT-PA	0	5	300 (3)	20.3	0	0	0	10.3	14.7	25.0	6.7
	20	5	200 (2)	33.0	0	0	1.5	51.5	49.0	102.0	6.0
	50	5	400 (4)	38.2	0	0	2.7	67.7	62.7	132.2	48.2
	100	5	200 (2)	46.0	0	0	5.0	126.0	66.5	200.5	4.9
N-SW	0	5	300 (3)	16.7	0	0	0	4.3	15.0	19.3	4.0
	20	5	200 (2)	26.0	0	0	0	8.5	26.0	34.5	7.5
	50	5	400 (4)	23.0	0	0	0	33.0	33.0	65.2	25.8
	100	5	200 (2)	27.5	0	0	2.0	15.5	30.5	48.0	7.1
AT-PA	0	24	300 (3)	16.3	0.3	2.3	0.3	4.0	12.0	18.3	4.6
	20	24	200 (2)	25.5	2.5	7.5	7.5	20.0	15.0	52.5	1.5
	50	24	400 (4)	37.7	3.5	7.7	6.5	19.2	32.2	71.7	4.9
	100	24	200 (2)	42.5	6.5	12.0	17.0	45.0	26.5	107.0	3.0
	200	24	200 (2)	47.0	6.0	9.0	16.5	51.0	42.5	125.0	9.0
N-SW	0	24	300 (3)	13.3	0	1.0	0	3.0	11.0	15.0	4.5
	20	24	200 (2)	15.0	3.0	5.0	0	6.0	8.0	22.0	3.0
	50	24	300 (3)	24.3	8.0	7.0	0	4.7	11.3	35.3	14.6
	100	24	200 (2)	21.5	3.5	1.5	0	8.0	17.0	30.0	2.0
	200	24	200 (2)	34.5	17.5	5.5	0	9.0	26.5	58.5	2.5

[a]Number of experiment in parenthesis.
[b]Including gaps.

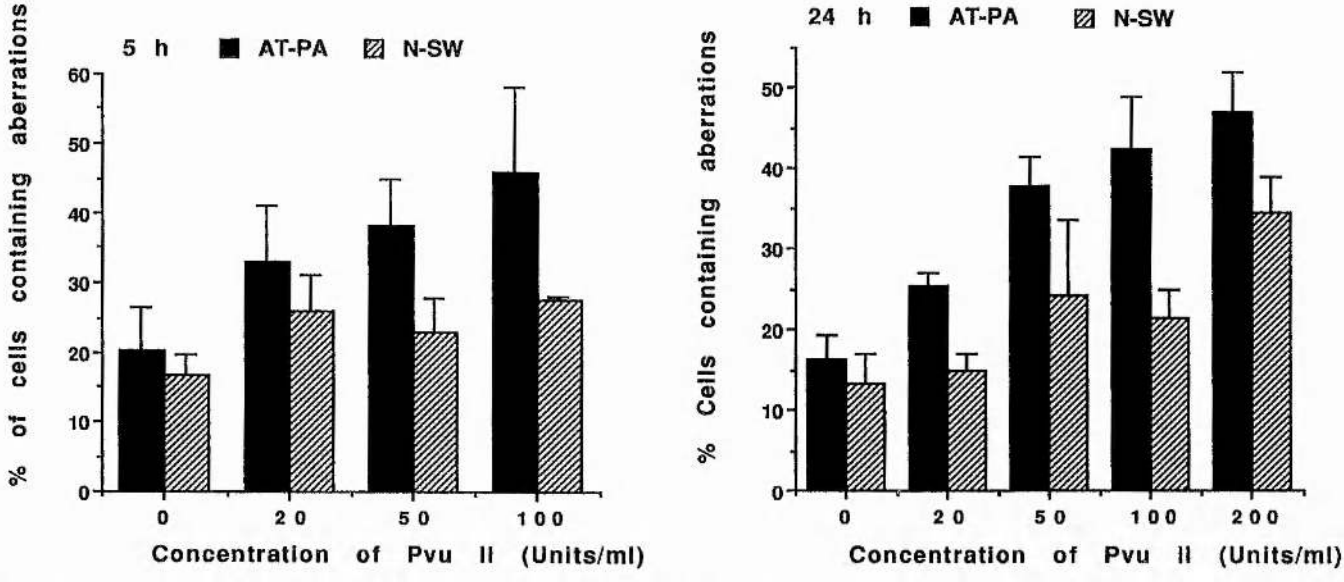

Fig. 7. Percent of cells containing chromosomal aberrations after (a) 5 h and (b) 24 h incubation in *Pvu*II treated AT-PA and N-SW cells. Error bars represent standard error of mean values (see Table II).

The fact that chromatid aberrations are induced by treatment in G_1 phase (assumed from the late sampling time used and the concurrent presence of chromosome aberrations) probably indicates persistence inside cells of the enzyme (*Pvu*II) into the S-phase. We have shown that *Pvu*II is a very stable enzyme; retaining almost full activity for >24 h at 37°C *in vitro* (data not shown) and therefore cutting by the enzyme is likely to continue for some hours after treatment. In the case of radiation it was hypothesized that long lived DNA lesions (e.g. base damage) might be responsible for chromatid aberrations induced in S phase (Taylor, 1978).

Our results in Figure 8 show that both normal (N-SW) and AT cells exhibit very low frequencies of chromosomal aberrations (breaks, gaps and exchanges) when treated with *Bam*HI, as was found previously for CHO cells (Bryant, 1984; Bryant and Christie, 1989). Although AT cells treated with *Bam*HI show a higher frequency of chromosomal aberrations than normal cells, the total number of aberrations was much lower when compared with *Pvu*II-treated cells. Two factors may possibly contribute to the lower yield of chromosomal aberrations caused by *Bam*HI: (i) the production of dsb by the enzyme, i.e. the activity and stability of *Bam*HI under cellular conditions and (ii) the processing of dsb caused by *Bam*HI.

Plasmid assay of *Bam*HI and *Pvu*II following purification was

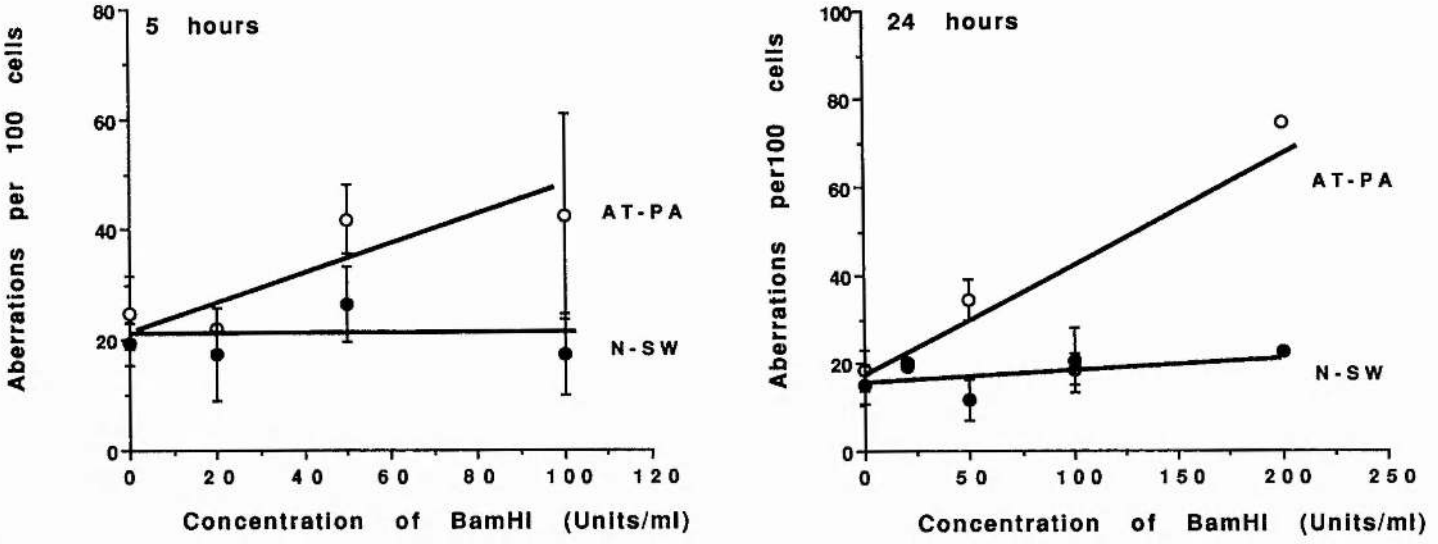

Fig. 8. Frequency of chromosomal aberrations (including gaps) in AT-PA (○) and N-SW (●) cells treated with *Bam*HI for (**a**) 5 h and (**b**) 24 h. Error bars represent standard error of mean values (see Table II).

Table II. Metaphase aberrations per 100 AT-PA or N-SW cells treated with *Bam*HI[a]

Cell line	Units/ml	Fixation time (h)	No. of cells analysed[b]	Percent of damaged cells	Chromosome exchanges	Chromosome deletions	Chromatid exchanges	Chromatid deletions	Chromatid gaps	Total aberrations[c]	SEM
AT-PA	20	5	200 (2)	16.5	0	0	0	8.0	14.0	22.0	0
	50	5	300 (3)	23.3	0	0	0	16.0	26.0	42.0	6.2
	100	5	200 (2)	16.5	0	0	3.0	25.0	14.5	42.5	18.5
N-SW	20	5	200 (2)	14.0	0	0	0	3.5	14.0	17.5	8.5
	50	5	400 (4)	17.0	0	0	0	11.5	15.0	26.5	6.7
	100	5	200 (2)	13.0	0	0	0	4.0	13.5	17.5	7.5
AT-PA	20	24	100 (1)	15.0	1	3	0	1	15	20	–
	50	24	200 (2)	24.5	0	2.5	0	8.5	23.5	34.5	4.5
	100	24	200 (2)	15.0	0	3.5	0.5	7.5	7.0	18.5	3.5
	200	24	125 (1)	32.0	2.4	7.2	7.2	26.4	31.2	74.4	–
N-SW	20	24	100 (1)	12.0	2	3	0	8	6	19	–
	50	24	200 (2)	9.0	0	1	0.5	5.5	4.5	11.5	4.5
	100	24	200 (2)	17.5	2	2	0	5.5	11.0	20.5	7.5
	200	24	125 (1)	19.2	0.8	4.8	0	5.6	11.2	22.4	–

[a]Number of experiment in parenthesis.
[b]Control (0 units/ml): See Table I.
[c]Including gaps.

carried out in boiled whole (normal) cell extract which was used in an attempt to simulate the conditions inside the cells. The results (data not shown) showed that the minimum concentration required to cut pBR322 completely was 2- to 4-fold higher for *Bam*HI than for *Pvu*II, i.e. the activity of *Bam*HI was lower than that of *Pvu*II. However, the results also showed *Bam*HI was similarly stable to *Pvu*II (under the assay conditions used) at 37°C for up to 24 h. When taking account of the lower activity of *Bam*HI measured under these assay conditions our data show that *Bam*HI is still less effective than *Pvu*II in both AT-PA and N-SW cells. Our data (Figure 4) also show that accumulation of dsb caused by *Bam*HI is much lower than that caused by *Pvu*II in N-SW cells. Therefore, we suggest, as we have previously (Bryant, 1984; Bryant and Christie, 1989), that cells may process cohesive-ended dsb more efficiently than blunt-ended dsb, producing a lower frequency of chromosomal aberrations.

Our results with *Bam*HI showing enhanced frequencies of aberrations in AT-PA over N-SW cells (Figure 8 and Table II) suggest that AT-PA cells either accumulate more dsb than N-SW cells do and thus perhaps convert a higher number of cohesive-ended dsb into chromosomal aberrations, or they may convert some of the cohesive-ended dsb induced by *Bam*HI into blunt-ended dsb, thus causing an enhanced level of chromosomal aberrations, by the same (or a similar) mechanism to that proposed by Cox *et al.* (1984) for AT5BIVA fibroblasts. In this mechanism cohesive-ended dsb with a 4 base 3′ overhang (incised by *Kpn*I) in transfected plasmid vector (pSVgpt) were reported to undergo exonuclease end-degradation, resulting in the loss of base sequence in the *gpt* gene, and possibly yielding a blunt-ended dsb prior to ligation (Cox *et al.*, 1984, 1986; Debenham *et al.*, 1987).

In normal N-SW cells aberration frequencies after 100 units/ml

(5 h sampling time) and 200 units/ml (24 h sampling time) *Bam*HI treatment were not significantly above the control (untreated) values. N-SW cells treated with *Bam*HI may ligate the cohesive dsb at a sufficient rate to preclude accumulation of dsb and a significant level of end degradation. In contrast there have been other reports that *Bam*HI and other restriction endonucleases causing cohesive-ended dsb are as effective as those causing blunt-ended dsb (e.g. Gustavino *et al.*, 1986; Winegar and Preston, 1988). However it should be borne in mind that high concentrations of *Bam*HI were used as well as poration techniques involving hypertonic shock to cells (see discussion in Bryant and Christie, 1989). It has been shown that *Eco*RI also induces significant numbers of aberrations in electroporated AA8 and EM6 cells (Cortes and Ortiz, 1992).

We conclude that AT-PA cells are characterized by a DNA dsb processing defect that converts higher numbers of blunt- or cohesive-ended dsb into chromosomal aberrations than in the normal N-SW line, and may also convert a fraction of cohesive-ended dsb into blunt-ended dsb so rendering them more clastogenic. Our results therefore support the notion that AT cells are hypersensitive to ionizing radiation as a result of a dsb processing defect.

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HYPER-SENSITIVITY OF ATAXIA TELANGIECTASIA CELLS TO DNA DOUBLE STRAND BREAKS INDUCED BY RESTRICTION ENDONUCLEASE

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Restriction endonuclease (RE) exclusively induces double strand breaks (dsb) in DNA. Therefore they have been widely used to study the relationship of dsb and chromosomal aberration (CA) in mammalian cells. We attempted to introduce RE into human lymphoblastoid cell lines derived from an ataxia telangiectasia homozygote individual (AT-PA) and a normal donor (N-SW), using streptolysin O poration. AT-PA cells exhibited about a 3-fold increased sensitivity to X-irradiation than N-SW cells when assayed by the micronucleus assay by the cytokinesis-block technique. The frequencies of CA induced by PvuII (causing blunt-ended dsb) showed an enzyme concentration-dependence in both cell lines, but was about a 5-fold higher frequency in AT-PA cells than in N-SW cells, at both 5h and 24h sampling times. The enhanced frequency of CA in AT cells was principally a result of an increase of chromatid aberrations, rather than chromosome aberrations at 24h sampling time. Interestingly, higher frequencies of chromatid exchanges appeared in AT-PA than in N-SW cells. BamHI (causing 4 base overlapped dsb) was also found to cause higher frequencies of CA in AT-PA but at a much lower rate than PvuII. The results suggest that AT-PA cells are characterised by a dsb processing defect that converts a higher number of dsb into chromosomal aberrations than in the normal N-SW line.

Enhancement of restriction endonuclease-caused chromosomal aberrations by 9-β-arabinofuranosyladenine in normal human and ataxia-telangiectasia lymphoblastoid cells

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9-β-Arabinofuranosyladenine (araA) is a potent inhibitor of DNA synthesis and was found to potentiate the killing effect of X-rays in mammalian cells. We have shown previously that araA enhances chromosomal aberrations caused by X-rays in G_2 normal human and ataxia-telangiectasia (AT) fibroblastoid cells. Here we report that araA has a similar enhancing effect on frequencies of chromosomal aberrations caused by restriction endonuclease (RE) in normal human and AT lymphoblastoid cells. PvuII which causes blunt-ended dsbs, and PstI which causes cohesive-ended dsbs, have been introduced into two AT lymphoblastoid cell lines (AT-PA and AT-KM) and a normal human lymphoblastoid cell line (N-SW), porated by streptolysin. The results showed that chromatid aberrations (deletions, gaps and exchanges) in the cells fixed at 5 h after PvuII or PstI treatment occurred at a higher frequency in both AT-PA and AT-KM than in N-SW. Incubation of RE treated cells with araA caused an enhancement of chromatid aberrations in AT-PA and AT-KM by a factor of about two. In N-SW, the frequencies were increased by a factor of 4·7 for PvuII and 2·8 for PstI. The enhancement ratio of araA for RE-treated AT and normal cells were higher than for γ-irradiated cells. The results suggest that the repair of chromosome damage caused by RE in normal cells is more sensitive to araA than in AT cells, and the inhibition by araA of chromosomal damage caused by RE is more effective than that caused by γ-irradiation in AT and normal cells.

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Radiosensitive Chinese hamster *irs*2 cells show enhanced chromosomal sensitivity to ionizing radiation and restriction endonuclease induced blunt-ended double-strand breaks

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The Chinese hamster *irs*2 cell line shows cellular hypersensitivity to ionizing radiation although the induction and repair of double-strand breaks (dsb) in bulk DNA is normal. Here we report that *irs*2 shows chromosomal hypersensitivity to ionizing radiation and the restriction endonuclease *Pvu*II. The ratio of induced chromosomal aberrations in *irs*2 versus V79 was similar to that for survival (factor of between 2 and 4). *Pvu*II was administered during cell poration with the bacterial toxin streptolysin O. We also report that when streptolysin O porated *irs*2 and V79 cells were treated with *Pvu*II, and dsb assayed by neutral filter elution, equivalent numbers of dsb were induced in the two lines as a function of time following treatment. Our data show that *irs*2 has a DNA damage processing defect that leads to enhanced conversion of blunt-ended dsb into visible chromosomal damage.

Introduction

A number of mutant Chinese hamster cell lines have been isolated that show increased sensitivity to ionizing radiation. To date, at least seven complementation groups for radiation sensitivity in Chinese hamster cells have been identified (Jones *et al.*, 1988; Jeggo *et al.*, 1991; Thacker and Wilkinson, 1991). Some of these mutants appear to be radiosensitive as a result of a deficiency in DNA double-strand break (dsb) repair, e.g. *xrs* mutants (Kemp *et al.*, 1984) and the XR-1 mutant (Giaccia *et al.*, 1985), whereas other mutants such as the *irs* group (*irs*1, 2 and 3) show apparently normal induction and kinetics of repair of dsb following irradiation, but nevertheless show a 2- to 3-fold elevated radiosensitivity (Jones *et al.*, 1987; 1990; Thacker and Ganesh, 1990). In this respect the *irs* mutants appear to resemble the cells of human ataxia telangiectasia individuals (Lehmann and Stevens, 1977).

The *irs* mutants (*irs* 1, 2 and 3) have been shown to belong to three different complementation groups (Jones *et al.*, 1988), and *irs*2 has been shown to belong to the same complementation group as the radiosensitive V79 mutants: V-C4, V-E5 and V-G8 (Zdzienicka *et al.*, 1989; Thacker and Wilkinson, 1991).

In addition to their ionizing radiation hypersensitivity, *irs*1 cells have been found to express an unusual cross-sensitivity to UV light (2- to 3-fold higher sensitivity), and enhanced sensitivity to ethyl methane sulphonate (EMS; 10-fold higher sensitivity) and mitomycin C (MMC; 60-fold higher sensitivity). *Irs*2 cells, however, showed near-normal sensitivity to UV and MMC, and only 2- to 3-fold higher sensitivity to EMS (Jones *et al.*, 1987). Both *irs*1 and 2 have been reported to be hypersensitive to the topoisomerase I inhibitor camptothecin (Thacker and Ganesh, 1990; Jones *et al.*, 1992).

The *irs*1, 2 and 3 mutants have been examined for an abnormal response of DNA synthesis to radiation (Jones *et al.*, 1990; Thacker and Ganesh, 1990). It was found that while *irs*1 and 3 showed a similar dose-dependent inhibition of DNA synthesis to the wild-type, *irs*2 showed radioresistant DNA synthesis, similar to that observed in ataxia telangiectasia, where an altered response of DNA synthesis to ionizing radiation has been attributed to lack of a normal inhibition of replicon inititiation (Houldsworth and Lavin, 1980; Painter and Young, 1980). The V-C4, V-E5 and V-G8 mutants (Zdzienicka *et al.*, 1989), like *irs*2, similarly show ataxia telangiectasia type radioresistant DNA synthesis.

Recently Cheong *et al.*, (1992) have shown that X-irradiated *irs*2 cells exhibit a prolonged G_2 delay as compared to the wild-type V79 line. However this seems unlikely to be related to the radiosensitivity of the line since these authors showed *irs*1 to have a *reduced* G_2 delay.

The *irs*1 mutant has been characterized cytogenetically by Tucker *et al.* (1991) and shown to yield both high spontaneous and radiation-induced frequencies of chromosomal aberrations, and cells unusually showed chromatid deletions after irradiation in the G_1 phase of the cycle, reminiscent of the chromosomal response of ataxia telangiectasia cells (Taylor, 1978).

The DNA damage processing defect(s), responsible for the enhanced cellular radiosensitivity of the irs mutants have not yet been identified. Experiments using vector-mediated dsb repair analysis in which cells were tested for the fidelity of rejoining restriction endonuclease cuts in plasmids (Debenham *et al.*, 1988) demonstrated that while *irs*1 showed a 3-fold reduced ability compared with the wild-type to correctly rejoin a restriction endonuclease induced dsb in a transferred selectable SV40–*Escherichia coli gpt* recombinant gene, *irs*2 cells showed normal ability to correctly join the dsb. Thus although the reduced dsb rejoining fidelity might contribute to the radiosensitivity of *irs*1, the reasons for the radiosensitivity of the *irs*2 mutant have not yet been identified.

In order to test the putative involvement of a dsb processing defect in the radiosensitivity of *irs*2, we have examined the cytogenetic response of *irs*2 and its parental V79 line to ionizing radiation and restriction endonuclease induced dsb.

Treatment of permeabilized cells with restriction endonucleases (Bryant, 1984) causes specific types of dsb in the absence of other types of lesions that characteristically occur when cells are exposed to ionizing radiation, and which may complicate an analysis of the relationship between dsb and chromosomal aberrations. This approach enables an examination of the question of whether a mutant cell line has a defect in the processing of dsb. For example we previously showed that the Chinese hamster *xrs*5 mutant line yielded higher frequencies of chromosomal aberrations than the wild-type CHO parental line when treated with either *Pvu*II or *Bam*H1 (Bryant *et al.*, 1987). We also showed, using the neutral filter elution technique that dsb accumulated to a higher level in *xrs*5 than in wild-type CHO cells when treated with *Pvu*II, confirming the dsb repair defect in this

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cell line (Costa and Bryant, 1991). We have interpreted the dsb kinetics following *Pvu*II treatment in terms of a competition between cutting of DNA by the restriction endonuclease and repair of dsb (Costa and Bryant, 1990).

Here we report on experiments in which we have treated *irs*2 and its parental V79 cell line with *Pvu*II using a recently developed technique for cell poration that utilizes the bacterial toxin streptolysin O (SLO; Bryant, 1992) and examined the frequencies of induced chromosomal aberrations and dsb by neutral elution. SLO has some important advantages over electroporation as a means of cell poration. For example, the recovery of cells after treatment was shown to be much higher (>90%) than after corresponding treatments with electroporation (33%). The technique also requires no special apparatus. We have shown that in Chinese hamster CHO cells, chromosomal aberrations are induced at very low concentrations of restriction endonuclease, as a linear function of enzyme concentration (Bryant, 1992).

Materials and methods

Cell culture

The Chinese hamster *irs*2 cell line and its parental V79-4 (kindly supplied by Dr J.Thacker, MRC Radiobiology Unit, Chilton, UK) line were routinely passaged in Eagle's minimal essential medium (MEM) with 10% fetal calf serum, essential amino acids and antibiotics. For experiments, exponentially growing cells were seeded at 10^6 cells per 75 cm^2 flask and incubated for ~24 h before use.

Irradiation

Cells were either X-irradiated using a Siemens Stabilipan set operated at 240 kV and 14 mA or were gamma irradiated in a ^{137}Cs IBL 437C gamma-irradiator (CIS UK Ltd., High Wycombe, Bucks, UK) at a dose rate of ~4 Gy/min. Irradiations were carried out in air in medium at ambient temperature (~20°C). Dosimetry was carried out with a ferrous sulphate method (Frankenberg, 1969).

Restriction endonuclease purification

In all experiments *Pvu*II was purified free of storage buffer using Amicon ultrafiltration (Bryant and Christie, 1989). After purification, *Pvu*II was diluted in Hank's balanced saline solution (HBSS) containing 1% bovine serum albumin (BSA) and 6 mM $MgCl_2$ to 1 U/ml.

Cell poration using SLO

The full details of this method have been published elsewhere (Bryant, 1992). Briefly cells were trypsinized, resuspended in MEM and centrifuged. Medium was aspirated and cells resuspended in HBSS−BSA at 10^6 cells/ml. *Pvu*II was added at various concentrations and SLO (stock solution of 1.9 U/ml stored at −20°C in 0.5 ml aliquots) was added to 0.5−1 ml samples of cells to a final concentration of 0.045 U/ml. After gentle mixing cells were held at ambient temperature (~20°C) for 5 min before adding 5 ml MEM and centrifuging. Supernatants were aspirated, cells resuspended in MEM and transferred to dishes for various times, either in the presence of 3 μg/ml cytochalasin B (in the case of micronucleus assay) or before addition of colcemid (0.04 μg/ml) before harvesting for metaphase chromosome assay.

Sample preparation

Metaphases. After colcemid treatment cells were trypsinized, centrifuged and resuspended in 0.075 M KCl for 10 min at room temperature before recentrifugation and resuspension in fixative (methanol:acetic acid, 3:1). After four washes in fixative, storage overnight at 4°C and a further two washes in fresh fixative, metaphase chromosomes were spread by dropping (in fresh fixative) on to ice-cold wet slides (in distilled water), rapidly flame dried and stained for 10 min in 3% Giemsa in cold tapwater.

Micronucleus assay. After incubation for 24 or 30 h in cytochalasin B containing medium, which was found to be an optimal time for sampling (Bryant, 1992), cells were harvested by trypsinization, cytospun (Shandon Cytospin 2), dried, fixed in pure methanol, dried again and stained in filtered 10% Giemsa in cold tapwater for 20 min.

Measurement of DNA dsb

Cells (2×10^6) in a 75 cm^2 flask were labelled with [^{3}H]thymidine at 3.7 kBq/ml and 1 μM thymidine for 24 h, trypsinised and resuspended in MEM, and either centrifuged and resuspended in HBSS−BSA at 1×10^6 cells/ml and treated with *Pvu*II as described above, or exposed to gamma rays in air in MEM at room temperature. Dsb were measured by neutral filter elution (Bradley and Kohn, 1979) with some modifications. Proteinase K was omitted from the lysis solution as this was found not to influence the amount of DNA eluted. After lysis at 60°C for 1 h (Okayasu and Iliakis, 1989) samples were eluted with 40 ml of a buffer containing 0.02 M Tris and 0.05 M sodium EDTA, pH 9.6, over a period of ~16 h. To determine the total DNA eluted, samples were collected into flasks, weighed, and 5 ml mixed with 6 ml Optiphase (LKB Ltd, and counted for radioactivity in a scintillation counter.

Results

Induction and repair of DNA dsb

Figure 1 shows results obtained using neutral filter elution on irradiated cells. A linear dose−effect relationship was obtained between fraction of DNA eluted and dose, indicating a linear induction of dsb. There was no significant difference in the induction of dsb by gamma-rays in *irs*2 and V79 cells over the range of doses tested.

Figure 2 shows results of experiments in which cells were allowed to repair following gamma-irradiation. The data shows that there was no difference in the repair of radiation-induced dsb in the two lines, confirming the response of *irs*2 reported previously (Jones *et al.*, 1990; Thacker and Ganesh, 1990).

In Figure 3 we show the results of incubating cells for up to 3 h following poration and exposure to *Pvu*II. Dsb were found to accumulate as a function of time after treatment as previously reported in CHO cells (Costa and Bryant, 1990, 1991). The data in Figure 3 show that although there was more variability than in radiation experiments there was no significant difference in the accumulation of dsb with time in the two cell lines.

Induction of micronuclei

Figure 4(a and b) shows frequencies of micronuclei induced in cytokinesis blocked binucleate *irs*2 and V79 cells exposed to

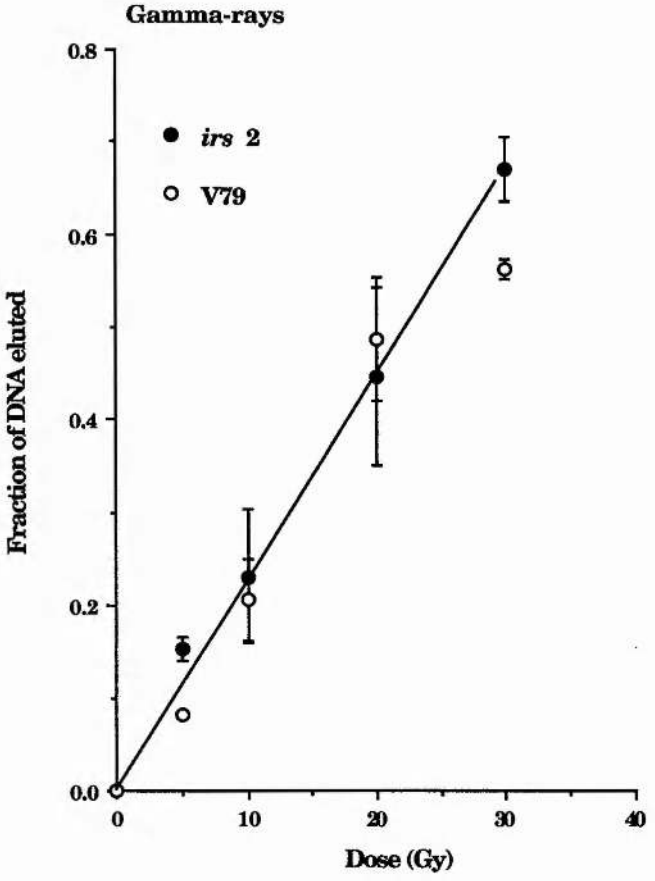

Fig. 1. Induction of DNA dsb as a function of dose in *irs*2 and V79 cells exposed to ^{137}Cs gamma rays and analysed by neutral filter elution. Results of two independent experiments. Error bars represent SEM.

X-rays and sampled at either 24 or 30 h. The results show that the frequencies of micronuclei in *irs*2 at both sampling times was approximately twice that in V79 cells. These sampling times were chosen as they were shown to be near the optimal sampling time for micronuclei in restriction endonuclease treated Chinese hamster cells (Moses *et al.*, 1990; Bryant 1992).

Induction of metaphase aberrations by gamma-rays

Figure 5(a) (and Table I) shows data for experiments in which cells were exposed to gamma-rays, and the frequencies of metaphase chromosomal aberrations scored at 2 and 18 h (Table I). In all cases aberration frequencies in *irs*2 were higher by a factor of between 3 and 4 than in V79. At 18 h the difference in aberration frequencies in *irs*2 and V79 was less, but still higher in *irs*2 by a factor of 2.5 than in V79.

Highest aberration frequencies were observed at the 2 h sampling time (representing G_2 cells), even though half the radiation dose was used, and the aberration frequency in *irs*2 was found to be 3.5 times higher than in V79.

The occurrence of chromatid aberrations in the scores of cells sampled at 18 h (presumed to be largely in the G_1 phase at the time of irradiation) was noted, particularly in *irs*2 cells.

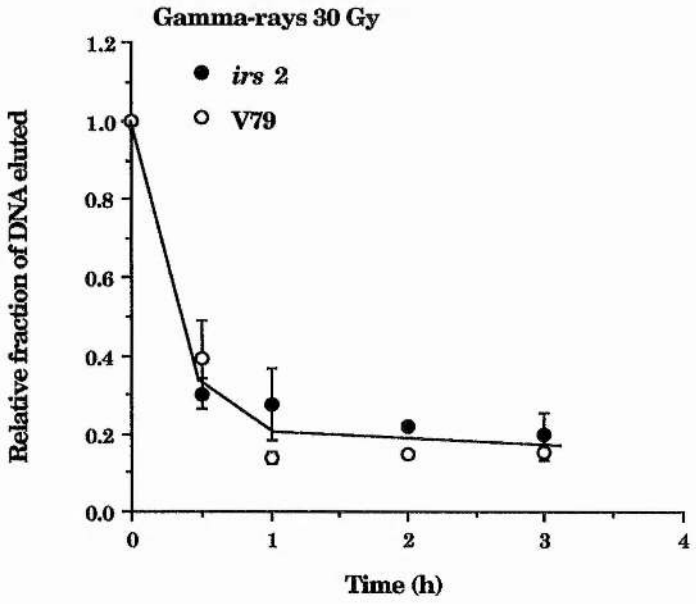

Fig. 2. Repair of DNA dsb in *irs*2 and V79 cells, as a function of time after exposure to ^{137}Cs gamma-rays. Results of two independent experiments. Error bars represent SEM.

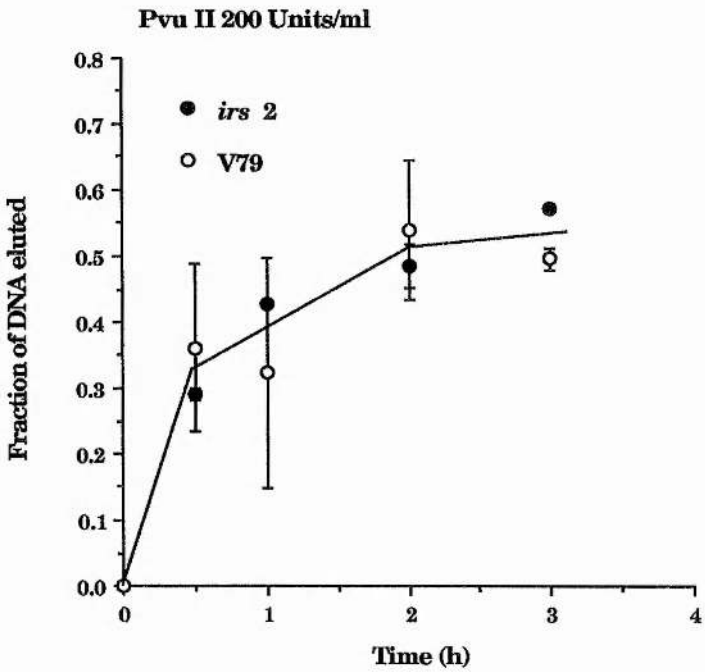

Fig. 3. Induction of DNA dsb in *irs*2 and V79 cells, as a function of time after poration and exposure to the restriction endonuclease *Pvu*II. Results of two independent experiments. Error bars represent SEM.

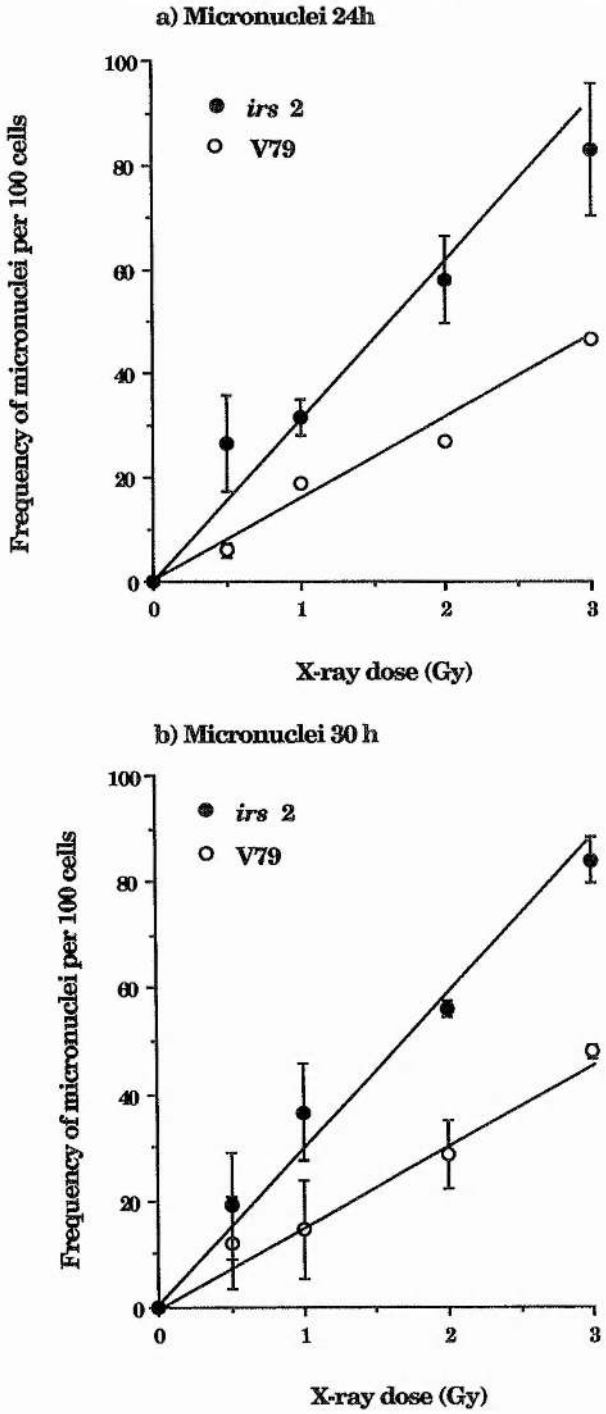

Fig. 4. Induction of micronuclei in cytokinesis-blocked (binucleate) *irs*2 and V79 cells (a) 24 h and (b) 30 h after exposure to X-rays. Results of two independent experiments. Error bars represent SEM.

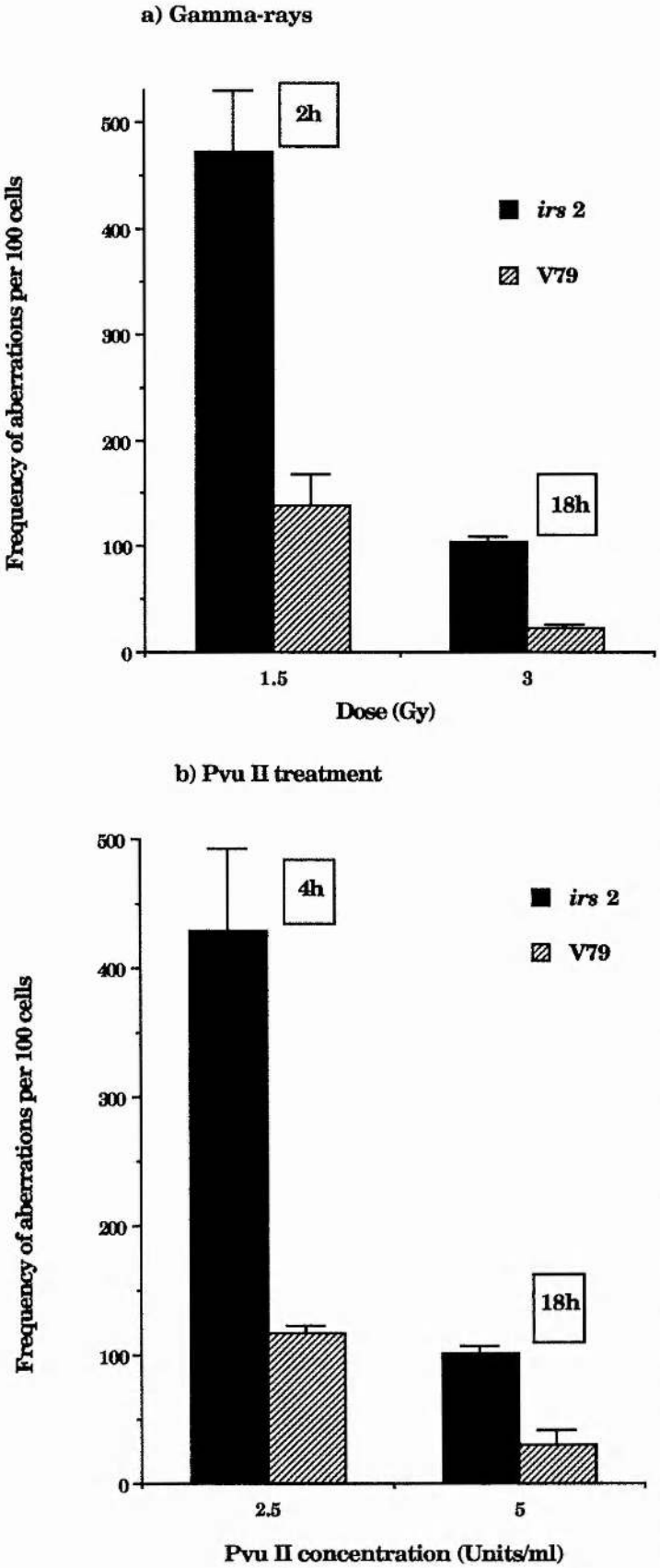

Fig. 5. Frequencies of total metaphase chromosomal aberrations in *irs*2 and V79 cells (a) at 2 and 18 h after exposure to ^{137}Cs gamma-rays and (b) at 4 and 18 h after poration and exposure to the restriction endonuclease *Pvu*II. In (a) and (b) note the different doses at the different times. Results of two independent experiments. Error bars represent SEM.

Induction of metaphase aberrations by PvuII

In Figures 5(b) and 6 (and Table II) we present results obtained for cells porated and treated with the restriction endonuclease *Pvu*II. The aberrations resulting from *Pvu*II treatment at the three sampling times used (4, 18 and 24 h) were of the same types as observed after gamma-irradiation. As for gamma-irradiation, the highest frequencies were observed at the shortest sampling time (4 h) which was chosen in order to accumulate sufficient metaphases to facilitate scoring. The aberrations scored at this time were of the chromatid type (gaps, breaks and exchanges). At 18 and 24 h aberration frequencies were lower, even at twice the enzyme concentration, and fortuitously, the doses of radiation and *Pvu*II selected yielded almost identical frequencies of aberrations. Frequencies of aberrations in *irs*2 were higher than those in V79 at the 4 h sampling time by a factor of 3.7, whereas at 18 and 24 h the difference between *irs*2 and V79 was 2- to 3-fold.

Figure 6 shows the data at 18 h sampling time plotted as a function of *Pvu*II concentration. A near linear increase in aberration frequency was observed for both *irs*2 and V79 cells.

Discussion

The results of neutral elution experiments on gamma-irradiated cells (Figure 1) confirm and extend those reported previously (Jones *et al.*, 1990; Thacker and Ganesh, 1990) in showing that the frequency of dsb induced in *irs*2 cells is not different from that in V79 cells. Similarly the repair kinetics of dsb in the two lines appear to be the same over the time intervals tested. Thus there appears to be no difference in repair of bulk dsb in the *irs*2 mutant that can be held as an explanation of the cellular hypersensitivity to ionizing radiation.

Our chromosome damage data in X- and gamma-irradiated *irs*2 and V79 cells (Figures 4a, b and 5a) shows that *irs*2 has an elevated chromosomal radiosensitivity paralleling its 2- to 3-fold cellular hypersensitivity to X-rays. Our micronucleus data with X-rays thus emphasizes the previously established strong association of chromosomal damage with cell killing (Joshi *et al.*, 1981).

Despite its increased chromosomal sensitivity, *irs*2 does not appear to exhibit a defect in the repair of ionizing radiation induced DNA ssb or dsb (Jones *et al.*, 1987, 1990; Thacker and Ganesh, 1990; Cheong *et al.*, 1992; this paper).

These data are analogous to those found for the mutant *irs*1 and ataxia telangiectasia cells. It has been postulated that *irs*1 cells may either misrepair DNA strand breaks or are defective in the repair of a crucial subset of strand breaks (Jones *et al.*, 1990; Tucker *et al.*, 1991) and that these then give rise to the observed elevation of chromosomal aberrations. Such a repair defect would not be detectable with assays such as neutral filter elution. Although the chromosomal aberration data for *irs*1 and *irs*2 are similar, there are subtle differences. *Irs*1 cells, when irradiated in G_2, primarily exhibit an increase in chromatid exchanges whilst *irs*2 shows an elevation of both exchanges and breaks. Following irradiation in G_1 all types of aberrations are increased in *irs*2 (chromatid and chromosome breaks and exchanges) whereas in *irs*1 the main increase is in chromatid aberrations (both exchanges and breaks). These data indicate that the DNA repair defects in *irs*1 and *irs*2 are distinct. One possible interpretation is that whilst *irs*1 may misrepair DNA damage (Debenham *et al.*, 1988; Jones *et al.*, 1990; Tucker *et al.*, 1991), *irs*2 may be defective in processing, or targeting repair to a subset of dsb, possibly located within active domains of DNA.

The fidelity of repair of dsb has been shown to be the same

Table I. Metaphase aberrations in *irs*2 and V79 cells exposed to gamma-rays[a]

Cell Line	Dose (Gy)	Fixation time (h)	No. of cells analysed	No. of cells damaged	Chromatid breaks[b]	Chromatid exchanges[c]	Chromosome breaks[d]	Chromosome exchanges	Total aberrations per 100 cells	SEM	Ratio[e]
V79	0	2	200	2	2	0	0	0	1.0	0	—
	1.5	2	200	140	263	16	0	0	139.5	29.5	—
*irs*2	0	2	200	16	14	3	0	0	8.5	3.5	—
	1.5	2	200	198	841	122	0	1	482.0	56.0	3.5
V79	0	18	200	11	11	0	0	0	5.5	0.5	—
	3	18	200	43	22	4	18	11	27.5	4.5	—
*irs*2	0	18	200	21	20	0	0	1	10.5	2.5	—
	3	18	200	120	77	26	80	47	115.0	4.0	4.8

[a]Pooled results of two independent experiments.
[b]Including gaps.
[c]Including triradials.
[d]Including isolocus breaks.
[e]Ratio of total aberrations *irs*2/V79.

in *irs*2 as in the wild-type V79 line, as tested by the joining of restriction endonuclease induced dsb in a transfected plasmid vector (Debenham *et al.*, 1988); the fidelity of repair in both V79 and *irs*2 was 18% for a *Kpn*I induced dsb (3′ overhang of four bases) and 36% for an *Eco*RV-induced dsb (blunt-ended) in plasmid pPMH16. However, this was in contrast to the response of the *irs*1 mutant that showed ~3-fold lower fidelity of repair of dsb than the wild-type V79 line (Debenham *et al.*, 1988).

Our findings might appear to be in conflict with those of Debenham *et al.* in that where they find no difference in joining fidelity of restriction endonuclease induced dsb (in a plasmid vector) between *irs*2 and V79, whereas we show here that *irs*2 does possess a defect in processing of restriction endonuclease-induced dsb. However, this difference probably reflects the different nature of the two assays used; one measuring the cell's ability to faithfully join a dsb in a transfected plasmid, i.e. its ability to restore the exact DNA code at the cut site, and the other measuring the frequency with which ends of dsb in genomic DNA are misjoined with one another or fail to be joined, thus converting the dsb into visible chromosomal aberrations.

Spontaneous levels of chromosome damage were not found to be elevated in *irs*2, as compared with V79, when using the micronucleus assay (results not shown). However, data in Table I shows that a significantly higher frequency of spontaneous metaphase chromosomal aberrations occurred in *irs*2 than V79, at both 2 h and 18 h sampling times. This finding is similar to that reported for the V-C4 and V-E5 mutants of Zdzienicka *et al.* (1989) where a 6- and 2-fold enhanced frequency of spontaneous aberrations was observed respectively.

The elevated frequency of gamma-ray induced aberrations in *irs*2 (Table I) also parallels the elevated levels seen in V-C4, V-E5 and V-G8, which are known to be in the same complementation group as *irs*2 (Zdzienicka *et al.*, 1989; Thacker and Wilkinson, 1991). However, as was found for spontaneous frequencies of aberrations in V-C4, V-E5 and V-G8, heterogeneity in the response of the V-mutants was observed, even though they are reported as belonging to the same complementation group.

That the chromosomal hypersensitivity of *irs*2 to radiation results from an altered processing of DNA dsb is supported by our data (Figures 5b and 6, and Table II) in which cells were treated with the restriction endonuclease *Pvu*II that causes blunt-ended dsb. The enhancement of chromosomal damage in *irs*2 was particularly evident (both for *Pvu*II and gamma-rays) in the G_2 phase of the cell cycle (Figures 5a and b, and Tables I and

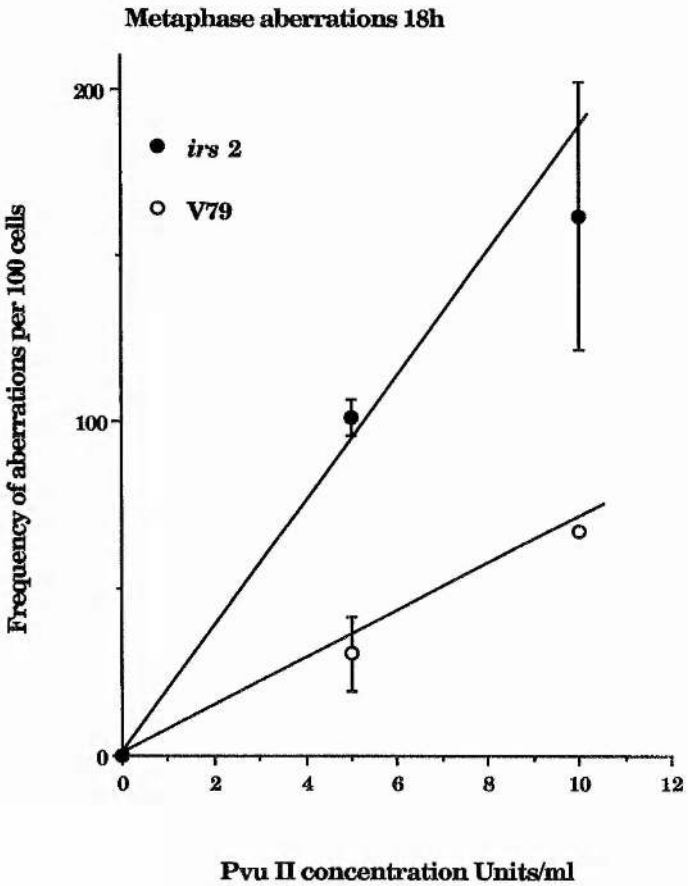

Fig. 6. Dose–effect curves for total metaphase chromosomal aberrations in *irs*2 and V79 cells 18 h after poration and exposure to the restriction endonuclease *Pvu*II. Results of two independent experiments. Error bars represent SEM.

II): cells showed ~3.5-fold higher aberration frequency than V79.

The occurrence of high frequencies of chromatid aberrations, particularly in *irs*2 cells sampled at 18 h after either gamma-rays or at 18 and 24 h after *Pvu*II indicates the possible persistence of lesions into the S-phase of the cell cycle. A similar effect was noted in the *irs*1 mutant by Tucker *et al.* (1991) and was likened to the response of ataxia telangiectasia cells (Taylor, 1978). In the case of *Pvu*II treatment this might possibly be attributed also

Table II. Metaphase aberrations in *irs*2 and V79 cells treated with *Pvu*II[a]

Cell line	Units/ml *Pvu*II	Fixation time (h)	No. of cells analysed	No. of cells damaged	Chromatid breaks[b]	Chromatid exchanges[c]	Chromosome breaks[d]	Chromosome exchanges	Total aberrations per 100 cells	SEM	Ratio[e]
V79	0	4	200	21	26	1	0	1	14.0	5.0	—
	2.5	4	200	81	202	57	0	2	130.5	6.5	—
*irs*2	0	4	200	29	49	0	1	1	25.5	3.5	—
	2.5	4	200	131	746	160	0	3	454.5	63.5	3.7
V79	0	18	200	37	34	9	0	0	21.5	6.5	—
	5	18	200	61	32	3	17	52	52.0	11.0	—
	10	18	200	70	54	26	15	83	89.0	1.0	—
*irs*2	0	18	200	13	12	0	2	0	7.0	2.0	—
	5	18	200	88	60	13	68	76	108.5	5.5	3.3
	10	18	200	108	112	113	59	54	169.0	40.0	2.4
V79[f]	0	24	100	13	10	4	0	0	14	—	—
	5	24	100	31	17	0	7	28	52	—	—
	10	24	100	48	23	1	27	58	108	—	—
*irs*2[f]	0	24	100	18	14	0	4	2	20	—	—
	5	24	100	41	37	5	24	36	102	—	2.2
	10	24	100	61	70	29	93	73	265	—	2.6

[a]Pooled results of two independent experiments (except f).
[b]Including gaps.
[c]Including triradials.
[d]Including isolocus breaks.
[e]Ratio of total aberrations *irs*2/V79 (after subtraction of controls).
[f]Results of a single experiment.

to persistence into S-phase of the enzyme inside the cell, since *Pvu*II is known to be very stable (S.A.Moses and P.E.Bryant, in preparation).

It is interesting to note that G_2 V79 cells were chromosomally less sensitive than wild-type CHO K1 cells, as determined in our previous studies (MacLeod *et al.*, 1990). For 1.5 Gy, G_2 V79 cells suffered almost four times fewer chromatid breaks than CHO and >2-fold fewer chromatid exchanges. Hence the ratio between breaks and exchanges was different in the two lines; in CHO for 512 breaks (induced by 1.5 Gy in G_2 cells) 18 exchanges were induced (~28 breaks per exchange), whereas in V79 for 132 breaks induced eight exchanges were induced (~16 breaks per exchange). This may be further evidence for the difference in the mechanisms of induction of G_2 breaks and exchanges, as previously postulated (Mozdarani and Bryant, 1987).

In Figure 3 we show that *irs*2 and V79 cells treated with *Pvu*II accumulate dsb at a similar rate indicating that penetration of the enzyme during poration, cutting frequency and dsb repair rate are similar for mutant and parental lines. We have previously interpreted this type of kinetic on a basis of a competition between cutting by *Pvu*II and cellular repair of the induced dsb (Costa and Bryant, 1990). This notion was supported by the finding that the X-ray sensitive *xrs*5 line of Chinese hamster cells (Jeggo and Kemp, 1983), defective in repair of dsb (Kemp *et al.*, 1986), showed an enhanced rate of accumulation of dsb with time as compared with its wild-type CHO K1 parental line (Costa and Bryant, 1991). The putative reduced ability of *xrs*5 to repair *Pvu*II induced dsb was also manifest as an increased induction by *Pvu*II of chromosomal aberrations in *xrs*5 cells (Bryant *et al.*, 1987).

We have postulated previously that the step between dsb and chromosome (or chromatid) breaks involves a conversion mechanism that appears to be largely independent of the rate of repair of dsb in bulk DNA. Such conversion of dsb into aberrations is evident from experiments with ataxia telangiectasia cells (Mozdarani and Bryant, 1989) where the rate of disappearance of G_2 chromatid breaks with time is similar in ataxia telangiectasia and normal human cells but the frequencies of breaks are 2- to 3-fold higher, even at short times (30 min) after exposure to X-rays. Similar findings have been reported recently using the premature chromosome condensation technique in ataxia telangiectasia and normal human cells (Pandita and Hittleman, 1992) where enhanced 'conversion' of dsb into breaks was similarly invoked as the mechanism for this chromosomal hypersensitivity.

The mechanism of conversion of dsb into visible breaks is not yet understood; however, it is possible that it might take the form of enzymatic changes to the ends of a small number of dsb so that they can no longer be rejoined. This could happen for example via the covalent closure of the termini of a few dsb. At mitosis the condensation of chromatin might then reveal the break as a visible break in the chromosome. Alternatively, the presence of a non-rejoinable dsb might prevent correct repackaging of chromatin at that point and thereby lead to a visible break in the chromosome or chromatid.

Although we have not yet established the kinetics of disappearance of chromatid breaks in G_2 *irs*2 and V79 cells, our data in Tables I and II indicate a higher conversion of dsb into chromatid breaks occurred in *irs*2 than in V79 after treatments with either gamma-rays or *Pvu*II.

We have shown for the first time that a mutant cell line, which when examined using conventional assays for dsb in bulk DNA (neutral velocity sedimentation, neutral filter elution and neutral pulse-field gel electrophoresis) Jones *et al.*, 1990; Thacker and Ganesh, 1990; Cheong *et al.*, 1992) exhibits an apparently normal ability to repair radiation-induced dsb, but is chromosomally hypersensitive to restriction endonuclease mediated dsb. We therefore suggest that the hypersensitivity of *irs*2 to ionizing radiation may result from a dsb processing defect that results in conversion of a higher proportion of dsb into chromosomal aberrations than occurs in V79. These

chromosomal aberrations are of both exchange and deletion types and result in the loss of large fragments of genetic material, subsequently leading to enhanced cell kill in *irs2*.

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